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# **FACTORS THAT CONTRIBUTE TO THE PATHOGENESIS OF DENGUE IN ADULT PATIENTS IN VIET NAM**

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*A Thesis submitted in partial fulfillment of the requirement of  
the Open University - United Kingdom  
for the degree of Doctor of Philosophy.*

**Oxford University Clinical Research Unit Viet Nam**

Submission date: 30 June 2008

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# ABSTRACT

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Dengue fever and dengue hemorrhagic fever (DF /DHF) are caused by the dengue viruses, which belong to family Flaviviridae. DHF incidence has been increasing globally in the last 40 years. Over half the world's population lives in areas potentially at risk for dengue transmission, making dengue the most important human viral disease transmitted by arthropod vectors in terms of morbidity and mortality. Recently, the age distribution of DHF cases in Asia has changed. The number of adults with DHF has increased since the 1990s in Southeast Asian countries including Viet Nam.

A series of studies were conducted in the Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam on clinical manifestations, cytokine production in acute infection and T cell responses in adult dengue patients.

A high proportion of adult dengue patients had bleeding and abdominal symptoms. DSS were observed in 45% of the in-patient study population. The existing WHO classification system and case definitions were inadequate to distinguish between adult Vietnamese patients with or without severe bleeding. Therefore, it is suggested that a reassessment of the dengue classification scheme is needed for use

by clinicians, epidemiologists, public-health authorities, vaccine specialists, and scientists involved in dengue pathogenesis research.

The in-vivo cytokine profile in dengue patients strongly implicated the role of vaso-active cytokines such as TNF $\alpha$ , soluble TNF receptors, IFN $\gamma$ , IL-2, IL-6, IL-10 and possibly, the imbalance between these proinflammatory and anti-inflammatory cytokines in the pathogenesis of dengue infection.

Six candidate HLA-A\*11 restricted dengue serotype 2 - specific CD8<sup>+</sup> T lymphocyte epitopes were identified with freshly collected PBMC from secondary dengue patients using motif-based predicted peptides. The cross reactivity of NS3<sub>133-142</sub> peptides against PBMC from patients infected with all four serotypes was studied further by using ELISpot assays and cell surface tetramer staining. Short-term CD8<sup>+</sup> CTL lines and clones were expanded by stimulating with the different variants of the immunodominant NS3<sub>133-142</sub> peptide from acute and convalescence PBMCs of DHF patients with secondary dengue. The functional phenotype of these cross-reactivity CD8<sup>+</sup> CTL lines and clones were then evaluated and characterized by cytotoxic activity and cytokine secretion. Our major finding was that highly cross-reactive CTL clones produce higher levels of inflammatory cytokines than serotype – specific clones and this might have implications in the pathogenesis of dengue. The fact that cross-reactive immune responses between dengue serotypes may play a part in the pathogenesis of severe disease should be considered in dengue vaccine development.



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## ACKNOWLEDGEMENTS

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The work described in this thesis would not have been possible without the participation of the dengue patient adults who admitted to the Hospital for Tropical Diseases of Ho Chi Minh City. I am indebted to them for their consent to take part in the studies described within this thesis.

I am enormously grateful to *Doctor Cameron Simmons* and *Professor Jeremy Farrar*, my supervisors, who have been a constant source of scientific, logistical and spiritual support. Without them, this thesis would never have been completed.

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The deepest thanks are expressed to those who have taken a skeptical view about my work. They have often pressured me to overcome all the inevitable difficulties to finish the studies described in this thesis.

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The financial support of this work from the Wellcome Trust of Great Britain is sincerely appreciated.

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# DECLARATION

---

Other than the assistance outlined in the acknowledgements, the work described in this thesis is my own work. I participated in designing, implementing, caring for all patients involved in the studies; taking and preparing blood samples (Chapter 3, 4, 5); performing the assays (Chapter 3, 4, 5). I have been involved in all the data analysis.

My work described in this thesis has not been submitted for a degree or other qualification to this or any other university.

The whole is more than the sum of its parts.

*Aristotle (ca 330 BC) Metaphysica 10f-1045a*

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## ABBREVIATION

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ADE:	antibody-dependent enhancement
ALT:	Alanine transaminase
AST:	Aspartate transaminase
CRF:	Case Record Form
D1V:	Dengue serotype 1 virus
DEN-1:	Dengue serotype 1
DF:	Dengue Fever
DHF:	Dengue Haemorrhagic Fever
DSS:	Dengue Shock Syndrome
Hct:	Haematocrit
HTD:	the Hospital for Tropical Diseases
IL:	Interleukin
INF:	Interferon
SD:	Standard deviation
TNF:	Tumor necrosis factor
WHO:	World Health Organization
95%CI:	95% confidence interval

---

# **CHAPTER 1**

## **INTRODUCTION**

---

Dengue fever and dengue hemorrhagic fever (DF/DHF) are caused by dengue viruses, which belong to the genus *Flavivirus*, family *Flaviviridae*. There are four antigenically related, but distinct, dengue virus serotypes (DEN-1, DEN-2, DEN-3 and DEN-4), all of which can cause DF /DHF. Classically, dengue virus infections cause a spectrum of illness ranging from asymptomatic, mild undifferentiated fever, to classical DF, dengue fever with haemorrhagic manifestations, or dengue hemorrhagic fever (DHF). This chapter reviews the history of dengue infection, the current situation of DF /DHF as "a global public health problem in the twentieth century", clinical manifestations of dengue infection and the pathogenesis of the diseases.

## **1.1. Dengue infections**

### **1.1.1 History of dengue**

Because dengue fever has nonspecific clinical features, the interpretation of historical records for evidence of past epidemics is open to speculation. The origin of the dengue viruses has been controversial. Some early authors speculated on an African origin and subsequent distribution around the world with the slave trade (Hirsch 1883) (Ehrenkranz, Ventura et al. 1971).

Dengue viruses are highly adapted to their mosquito hosts and responsible for forest cycles, with periodic amplification in lower primates. Such forest cycles have been documented in South-East Asia and Africa, and involve several species of lower primates and three subgenera (*Stegomyia*, *Finlaya* and *Diceromyia*) of canopy-dwelling mosquito species of the genus *Aedes* (Rudnick 1967) (Cornet

1993). Biologically, the persistence of dengue virus in the successive generations of mosquitoes infected through vertical transmission (i.e., from one generation to another) was reported with transmission rates (the percentage of parent females transmitting to one or more progeny) ranged up to 95% (Fauran, Laille et al. 1990) (Freier and Rosen 1988). These observations suggest that vector mosquitoes may play an important role in the maintenance of virus in nature, and that mosquitoes may act as reservoirs of dengue viruses (Joshi, Mourya et al. 2002).

A 1780 Philadelphia epidemic described by Benjamin Rush was perhaps the earliest description in English of so-called break-bone fever (Rush 1789). However, the earliest clinical and epidemiologic description of a potential dengue – like illness found to date is in a Chinese “encyclopedia of disease symptoms and remedies”, first published during the Chin Dynasty (AD 265 – 420) (Nobuchi 1979). The disease was called “water poison” by Chinese and was thought to be somehow connected with flying insects associated with water. It was characterized by fever, rash, eye pain, arthralgias, myalgias and hemorrhagic manifestations, including pharyngeal, gingival, intestinal and vaginal bleeding.

Subsequently, significant numbers of sporadic outbreaks of hemorrhagic disease were reported in continental United States through the early decade of the 20<sup>th</sup> century. Clinical descriptions of dengue complicated by hemorrhages, shock and death were reported in outbreaks in Australia in 1897, Greece in 1928, and Taiwan in 1931 (Hare 1898) (Copanaris 1928); (Akashi 1932).

Mosquito – borne transmission of the dengue infection by the *Aedes aegypti* mosquito was demonstrated in 1903 (Graham 1903). Sabin and his colleagues

isolated viruses from US soldiers in India, New Guinea and Hawaii in 1944 (Sabin 1945) (Sabin 1952)

After World War II, dengue cases complicated by haemorrhage and shock (DHF and DSS) were increasingly documented in Southeast Asia. Between 1954 and 1963 outbreaks of a newly recognized severe infectious disease of children were reported successively from the Philippines, Thailand, (Hammon, Rudnick et al. 1960), Singapore (Chew, Leng et al. 1961), and India (Ramakrishnan, Gelfand et al. 1964). In Viet Nam, Mihov and co-workers reported an outbreak of hemorrhagic fever in Hanoi, North Viet Nam, which appears to be the earliest observation of this disease on the east coast of the Dong Duong (Indo-China Peninsula). These authors reported clinical findings in 68 patients hospitalized at the Pediatric Clinic of the University of Hanoi during the rainy season of 1958. Although the total number of cases was unknown, it was estimated that many hundreds were involved. The disease affected children and was characterized by collapse, gastrointestinal hemorrhage and a mortality rate of 7%. Patients usually had thrombocytopenia, mild leukocytosis and hepatomegaly, but no urinary abnormalities. The authors did not mention whether dengue-like disease occurred simultaneously in the community (Mihov 1959).

According to available records and personal communication, Halstead et al. realised that the first recognized outbreak of hemorrhagic fever in South Viet Nam occurred between April and July 1960 in the town of Cái Bè, Tiền Giang Province. Cái Bè is located approximately 90 Kms southwest of Ho Chi Minh City (Saigon). Nearly 50 deaths were attributed to this outbreak. A few cases were admitted to the



Provincial Hospital at My Tho, but most cases were not hospitalized; thus, no accurate estimate of morbidity could be made (Halstead, Voulgaropoulos et al. 1965). The first virologically studied outbreak of mosquito-borne hemorrhagic fever occurred in the Mekong River delta (Cai Lây) and the city of Saigon, South Viet Nam, in the rainy season of 1963. Clinically, the disease resembled that described in other outbreaks in Southeast Asia. Three dengue viruses identified tentatively as type 2 were recovered, two from patients and one from *Aedes aegypti* captured in Saigon. Serologic data strongly suggested dengue virus etiology in clinically diagnosed cases (Halstead, Voulgaropoulos et al. 1965).

Epidemic activity of DHF intensified but remained confined to Southeast Asia through the 1970s. However, in the 1980s and 1990s, epidemic DHF spread west into India, Pakistan, Sri Lanka, and the Maldives and reemerged in China (Fan, Yu et al. 1989).

In the Americas, dengue was not considered a major public health problem until recent years. In 1981, the first major DHF epidemic in the Americas occurred in Cuba. During that outbreak, an estimated 344,000 dengue infections occurred and over 116,000 hospitalized patients and 10,000 cases of DHF were reported from May to October (Kouri, Mas et al. 1983). Moreover, by 1997, DHF had emerged as a disease entity in several major and many minor epidemics in tropical and subtropical countries of the Americas.

In summary, DHF incidence has increased in Southeast Asia, the Americas, Oceania and world widely over the last 40 years. The reasons for the dramatic global emergence of DF/DHF as a major public health problem in the twentieth

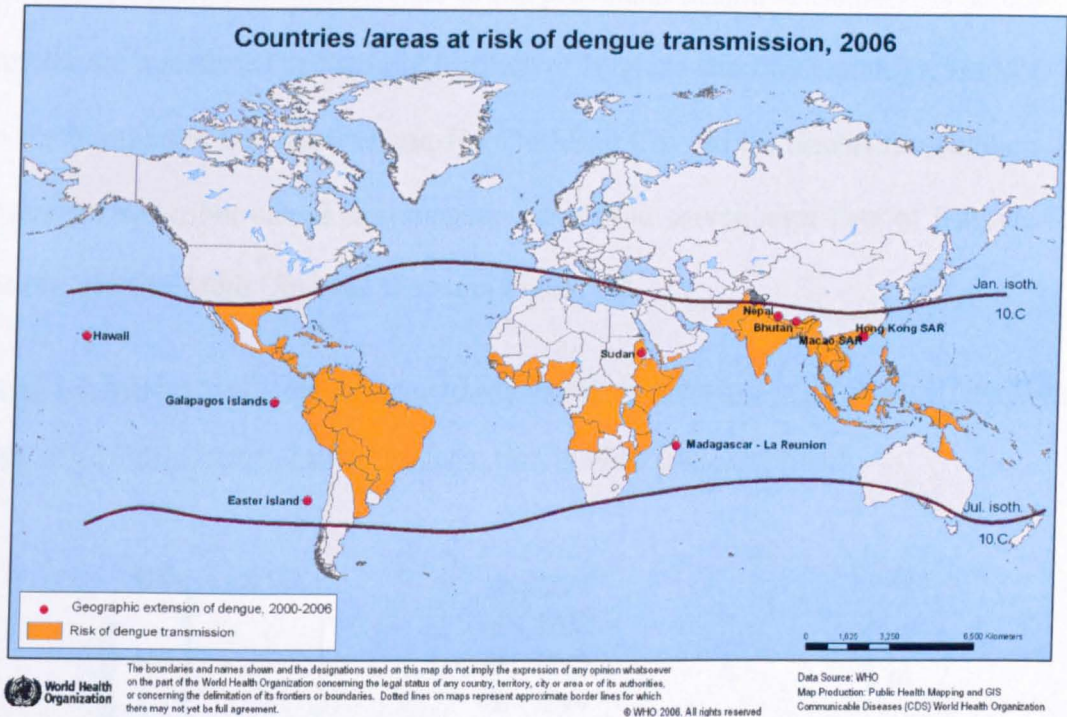
century are complex and not fully understood. However, several important factors can be identified, such as reinfestation of the American tropics by *Ae. aegypti* (Gubler and Trent 1993), ineffective mosquito control (Gubler 1989) (Newton and Reiter 1992), major global demographic changes with uncontrolled population growth, unplanned urbanization, and massive increase in global travel and trade particularly by airplane (Sutherst 2004) (Braks, Honorio et al. 2003) (Friel, McMichael et al. 2004).

### **1.1.2 Current dengue situation**

Today an estimated 50–100 million cases of dengue fever and 500,000 cases of DHF, resulting in around 24,000 deaths, occur annually, depending on the epidemic activity (Rigau-Perez, Clark et al. 1998) ((WHO) 1997). Over half the world's population lives in areas potentially at risk for dengue transmission, making dengue the most important human viral disease transmitted by arthropod vectors in terms of morbidity and mortality.

In South East Asia, the total number of reported cases and deaths from DF/DHF in nine countries of the SEA Region for the period 1985-2006 ranged from 46,458 (1986) to 218,821 (1998) and 188,684 (2006). Since 2003 the trend of reported dengue cases is rising although the case fatality rate is maintained below 1%. Unfortunately, despite the increase in the reported cases of DF/DHF, it is generally accepted that the incidence of the infection is largely under-reported (Gibbons and Vaughn 2002). Poor surveillance systems for dengue (Gubler 2002) and the non-specific clinical presentation were considered to be the reason for the underestimation of the infection (Hoang Lan Phuong 2006).

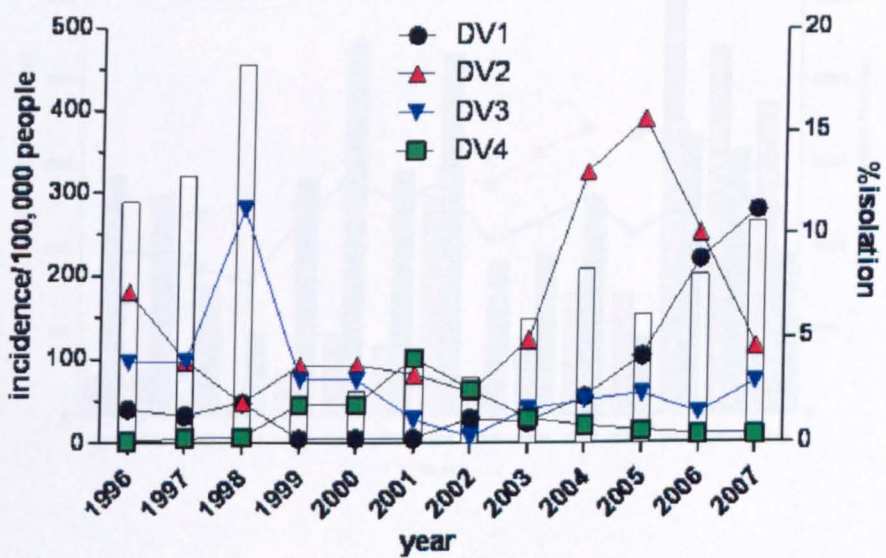
Figure 1.1: Dengue situation in the world (Source: World Health Organization - 10/Nov/2006)



The dengue disease burden in Viet Nam is almost certainly under-reported. The total disease burden is extracted from the routine health information system (HIS). The HIS of the public health services in Viet Nam reports at three levels: community, district and province. At community level, health data are recorded in a Health Examination Notebook (HEN) in which all patient consultations are being recorded. Routine surveillance of dengue is based on an algorithm supplied by the National Dengue Control Program that basically follows the guidelines of WHO, but does not require haematology support (haematocrit and/or platelets count). By using this algorithm, in principle, only dengue haemorrhagic fever and dengue shock syndrome are notified and uncomplicated dengue fever is not recognized.

Serological confirmation is only performed in the relatively small number of complicated cases that need referral to the provincial hospitals. Sometimes serum samples are transferred to National Institute of Hygiene and Epidemiology, Ha Noi (in the North) and Institute Pasteur, Ho Chi Minh City (in the South) for isolation of dengue virus, but not on a systematic basis. The surveillance data of Institute Pasteur, Ho Chi Minh City was shown in Figure 1.2.

Figure 1.2: Incidence of Dengue Haemorrhagic Fever and serotypes in the South of Viet Nam from 1996 - 2007 (Source: Pasteur Institute, Ho Chi Minh City, Viet Nam)



Result of study in central of Viet Nam, Binh Thuan province (total population = 1.12 million), in 2003 showed that every year, approximately 1.8% to 3.7% of the total population suffered from acute primary dengue, the total number of cases with acute primary or secondary dengue should range from 5.5% to 11.0% of the total

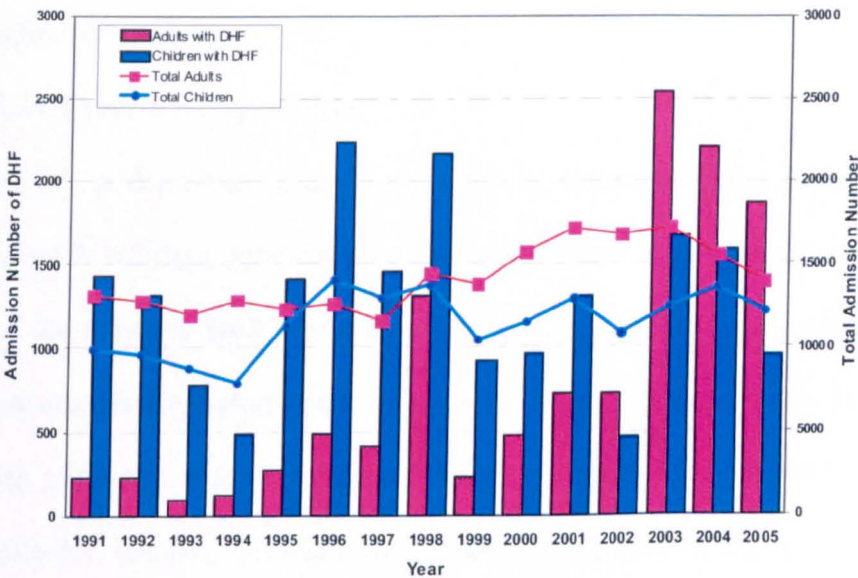


population (Hoang Lan Phuong 2006). Therefore, dengue is highly endemic in southern Viet Nam and leads to significant costs to the health care system.

1.1.3 Adult with Dengue infection at the Hospital for Tropical Diseases.

The number of DHF adult cases has increased during the 1980s and the 1990s in the Hospital for Tropical Diseases (HTD), Ho Chi Minh city, Viet Nam.

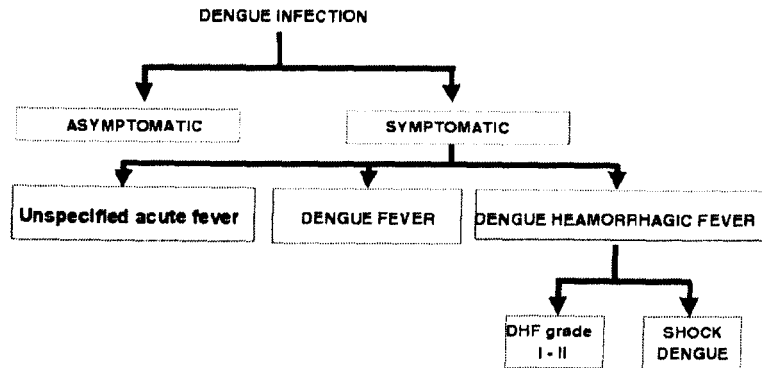
Figure 1.3: Dengue Haemorrhagic Fever in the Hospital for Tropical Diseases (Source: Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam)



1.2. Clinical Manifestation and Management of dengue infection

The World Health Organization has defined strict criteria for the classification of dengue, based on observations made in children during epidemics that occurred in Thailand during the 1970s (Fig. 1.4)((WHO) 1997).

Figure 1.4: Manifestation of the dengue syndrome (Source: CAB International 1997. Dengue and Dengue Haemorrhagic Fever. D.J. Gubler and G. Kuno)



### 1.2.1. Dengue Fever (DF)

#### 1.2.1.1 Clinical manifestation:

Classically, DF is described as a non-fatal febrile illness of older children and adults of about 5 to 7 days duration associated with sudden onset, extreme malaise, and pain of the muscles, back, limbs and eyes; rash is common as are other non-specific constitutional symptoms such as nausea, vomiting and headache. DF may be confused clinically with any other initially non-specific syndromes such as influenza measles, malaria, typhoid or leptospirosis. A definitive diagnosis of DF, therefore, can only be made by specific laboratory tests.

Dengue fever is associated with hemorrhage in 5 to 30 percent of cases. Variable degrees of bleeding may occur at any site, most commonly petechiae, purpura, epistaxis, gingival bleeding or menorrhagia, and usually on days 5 to 8 of the illness; hematuria, intracranial and gastrointestinal bleeding have also been reported in dengue fever. Unusually severe bleeding may cause shock and death due to blood loss (Hayes, Manaloto et al. 1988).

Other non-specific constitutional signs and symptoms which may occur include photophobia, drenching sweats, cough, sore throat, epistaxis, dysuria, hyperesthesia of the skin, pain in the groin and testicles, delirium, alteration in taste, lethargy, anorexia, lymphadenopathy and constipation or diarrhea (Neff, Morris et al. 1967) (Liu, Ho et al. 1989) (Chungue, Burucoa et al. 1992).

#### ***1.2.1.2 Laboratory findings***

Clinical laboratory findings associated with classic dengue fever include an early neutropenia with subsequent lymphocytosis, often marked by atypical lymphocytes. Leucopenia is seen in 50 to 90 percent of DF cases. Thrombocytopenia has been reported in 35 to 50 percent of confirmed cases (Dietz, Gubler et al. 1996). The WHO defines thrombocytopenia as a platelet count less than  $100,000 \text{ mm}^{-3}$  for DHF /DSS. Dietz et al. (1987) found that hemorrhage was associated with platelet counts below  $50,000 \text{ mm}^{-3}$ . The hematocrit and hemoglobin are normal in uncomplicated classical dengue fever. Likewise, prothrombin and partial thromboplastin times are normal and there are no fibrin split products present.

### **1.2.2. Dengue Haemorrhagic Fever (DHF)**

#### ***1.2.2.1 Clinical manifestation:***

Dengue hemorrhagic fever (DHF) is the most severe form of dengue infection with hemorrhagic diatheses and a tendency to develop shock (dengue shock syndrome - DSS) in addition to acute febrile illness ((WHO) 1997). The major pathophysiologic hallmarks that determine disease severity and distinguish DHF from DF are plasma leakage, due to an increase in vascular permeability, and

abnormal hemostasis. Hypovolemic shock occurs as a consequence of, and subsequent to, critical plasma volume loss selectively into serious spaces (i.e. pleural and peritoneal cavities). Abnormal hemostasis, including increased capillary fragility (positive tourniquet test and tendency to bruise), thrombocytopenia, impaired platelet functions and in the most severe form, disseminated intravascular coagulation (DIC), contributes to varying degrees of hemorrhagic manifestations. Typically, DHF is characterized by four major clinical manifestations, in order of their appearance and declining frequency as follows:

- High continuous fever of 2 to 7 days
- Hemorrhagic diathesis
- Hepatomegaly
- Circulatory disturbances (as shock in the most severe case)

Following the incubation period of 5 to 8 days, the illness usually begins abruptly with high fever accompanied by facial flushing, skin erythema, headache and muscle pain. The body temperature may reach 40 to 41°C, and febrile convulsions may occur, particularly in infants. Some patients may complain of sore throat, but rarely have rhinitis or cough. Anorexia, vomiting and abdominal pain are common. During the acute febrile phase, which usually lasts 2 to 7 days, DHF resembles DF in many respects. Hemorrhagic manifestations are usually mild and frequently found as tiny, scattered, petechial hemorrhages on the skin or occasionally in the buccal cavity and sub conjunctivae, and a tendency to bruise. Early in the febrile phase, a positive tourniquet test (more than 10 petechiae per 2.5 cm<sup>2</sup>) is frequently observed. Bleeding from the nose, gums and gastrointestinal tract may be severe.



Massive gastrointestinal hemorrhage may occur and is usually found later in association with prolonged shock.

The liver is often enlarged and palpable a few days after onset of fever. It is usually soft and tender, but jaundice is rarely observed (Agarwal, Kapoor et al. 1999). Splenomegaly is reported in 5 to 15% of cases.(Nimmannitya, Halstead et al. 1969) (Setiawan, Samsi et al. 1998) (Singh, Jhamb et al. 2005).

The critical stage is reached by the end of the febrile phase of illness. Accompanying or shortly after a rapid drop in temperature, varying degrees of circulatory disturbances develop. The patient is often sweating, restless and has cold extremities. In mild DHF cases, the changes in vital signs are minimal and transient, patients recovering spontaneously or shortly after a brief period of treatment. In more severe DHF cases, the disease progresses rapidly into a stage of shock. The onset of shock is acute and generally occurs at the time of defervescence, which is usually on or after the fourth day of illness and is acute. The patient usually complains of acute abdominal pain and becomes restless. Often with subnormal temperature, the skin is cold and clammy, and the pulse becomes rapid and weak. The pulse pressure is narrow ( $\leq 20$  mmHg), with characteristic high diastolic pressure (e.g. 100/90, 110/90 mmHg) in the early stage of shock. It is noteworthy that patients who are in shock usually remain conscious almost to the terminal stage. The course of shock is short, but life threatening. If proper resuscitation is not given, the patient deteriorates rapidly into the stage of profound shock, the pulse and/or blood pressure become undetectable, and usually die within 12 to 24 h after shock ensues. Prolonged shock is often complicated with metabolic

acidosis that may precipitate the occurrence of, or enhance the ongoing, DIC to the point that massive bleeding may occur. The most common severe bleeding is in the gastrointestinal tract, which usually presents as hematemesis (with dark-colored blood) and/or melena. The least common hemorrhagic manifestation is intracranial bleeding, but these patients have the highest fatality rates. (Sumarmo, Wulur et al. 1983) (George and Duraisamy 1981). Convulsions and coma are rare in patients with DHF in Thailand, but may be more frequently observed in other countries such as Indonesia and Malaysia (Lum, Lam et al. 1996).

Convalescence in patients with DHF, even in shock cases, is usually short and uneventful. Diuresis ensues as shock terminates and the patient rapidly regains appetite. Some patients may have a confluent petechial rash with characteristic, scattered, round areas of pale skin (without petechiae) on the extremities, more frequently on the lower extremities; occasionally it may be itchy. Bradycardia is a common finding during convalescence. This type of confluent petechial rash and bradycardia were originally described during convalescence in patients with DF (Sabin 1952). The course of uncomplicated DHF/DSS is about 7 to 10 days in most cases.

#### ***1.2.2.2 Laboratory findings***

Some significant laboratory changes occur in patients with DHF, and understanding these is critical for clinical diagnosis and management of the disease.

In the peripheral blood, normal white blood-cell counts or leucopenia with predominant neutrophils is common initially, and the majority of neutrophils are

young forms. Towards the end of the febrile phase, there is a reduction in the number of total white cells and neutrophils, simultaneously with an increase in the number of lymphocytes. A relative lymphocytosis with about 15 to 20 percent atypical lymphocytes is usually observed 1 to 2 days before defervescence (Thisyakorn, Nimmannitya et al. 1984).

Moderate to marked thrombocytopenia is a constant finding in DHF. The platelet count drops rapidly to a very low level ( $< 100,000 \text{ mm}^{-3}$ ) shortly before or simultaneously with a rise in hematocrit level, which reflects a leakage of plasma.

Hct determinations should be made more frequently after the platelet count has fallen. Clotting abnormalities are common, and laboratory evidence of DIC appears to be correlated with disease severity (Srichaikul, Nimmanitaya et al. 1977) (Srichaikul 1987).

Evidence of plasma leakage includes a rise in hematocrit leading to hemoconcentration during the period of leakage. The hematocrit levels could correlate with plasma volume loss and disease severity. However, the levels of hemoconcentration may be equivocal when there is frank hemorrhage early and excessive volume replacement, or untimely hematocrit determinations. Right-sided or bilateral pleural effusion is commonly found at the peak of plasma leakage by chest radiography. In cases of mild leakage, hydrothorax can be detected by right decubitus view. Hypoproteinemia/hypoalbuminemia and other evidence of plasma leakage are common, and a reverse albumin to globulin ratio is noted in about two-thirds of the patients (Nimmannitya 1987). Liver function tests show that most patients with DHF have elevation of aspartate aminotransferase (AST) and alanine

aminotransferase (ALT) (Liu, Ho et al. 1989) (Kuo, Tai et al. 1992) (Qiu, Gubler et al. 1993).

### **1.2.3. Clinical Diagnosis**

The severity of DHF has been arbitrarily classified into four grades according to the clinical hallmarks of shock and bleeding (Nimmannitya, Halstead et al. 1969). It is important to emphasize that cases are classified first as DHF with the presence of thrombocytopenia and concurrent hemoconcentration.

Grade I and grade II are non-shock DHF. In grade I the only hemorrhagic manifestation is a positive tourniquet test or tendency to bruise, while in grade II there is spontaneous bleeding, usually in the form of petechiae, ecchymoses, purpura, bleeding from the nose or gums, hematemesis and melena.

Grades III and IV are cases of DHF with shock (dengue shock syndrome). In grade III, signs of shock may include cold clammy skin, restlessness, rapid and weak pulse, narrow pulse pressure (20 mmHg or less) or hypotension. Grade IV cases are those with profound shock with undetectable pulse and/or blood pressure.

Occasionally, DF cases, particularly those with unusual hemorrhages or with thrombocytopenia, are misclassified as DHF grade I or grade II. It should be emphasized that thrombocytopenia with concurrent hemoconcentration or rising hematocrit are constant findings in DHF that differentiate non-shock cases of DHF from DF and other diseases.

### **1.2.4. Laboratory Diagnosis**

Laboratory diagnosis of dengue relies on the recovery of the virus by culture or on the detection of antibodies to the dengue virus ((WHO) 1997). Although culture is

the definitive diagnostic test, several practical considerations limit its use. First, virus isolation is mainly successful when attempted using clinical samples obtained during the acute phase of disease. Second, samples obtained for the detection of dengue virus by culture require proper handling. Third, not all laboratories are capable of culturing dengue viruses due to financial and safety reason.

Several promising laboratory techniques have been introduced in recent years with which either viral RNA or viral antigen can be detected in samples obtained from infected patients. Amplification of dengue RNA by reverse transcriptase polymerase chain reaction (RT-PCR) has proved successful (Lanciotti, Calisher et al. 1992) (Morita, Maemoto et al. 1994) (Warrilow, Northill et al. 2002). Using RT-PCR, dengue RNA in human samples can be quantified (Sudiro, Zivny et al. 2001). Recently, it was found that the nonstructural protein NS1 was circulating during the acute phase of infection (Young, Hilditch et al. 2000). Capture enzyme-linked immunosorbent assays (ELISAs) have been developed to detect the NS1 protein, and several reports indicate that detection of the NS1 protein may allow early diagnosis of infection (Young, Hilditch et al. 2000; Koraka, Burghoorn-Maas et al. 2003). Although an antigen detection kit is commercially available, and early studies suggest it is specific and reasonably sensitive, further studies are required to evaluate this assay are required (Koraka, Burghoorn-Maas et al. 2003) (Dussart, Labeau et al. 2006). Serological assays are most widely used in routine practice to confirm dengue virus infections and to differentiate between a primary and a secondary infection ((WHO) 1997; Vaughn, Green et al. 1997). Primary infections are characterized by an increase in the levels of dengue-virus-specific

immunoglobulin M (IgM) antibody 3–5 days after the onset of fever. IgM antibody titres continue to rise for 1–3 weeks and remain detectable for up to 6 months. Dengue-virus-specific immunoglobulin G (IgG) antibodies increase shortly after the initial rise in IgM antibodies to a modest degree and remain detectable for life ((WHO) 1997) (Vaughn, Green et al. 1997). In secondary infections, the IgM antibody level is generally lower than in primary infections, while IgG antibody levels rise rapidly from 1 to 2 days after the onset of symptoms (Vaughn, Green et al. 1997). The most commonly used serological techniques for the diagnosis of dengue infection are an ELISA that detects IgG or IgM antibodies and the haemagglutination-inhibition test. Traditionally, the haemagglutination-inhibition test has been used for the diagnosis of dengue and requires paired acute and convalescent sera collected 1 week or more apart for definitive diagnosis. However, this test requires samples to be pretreated, and the variability in methods used in different laboratories has compromised its general applicability. Today, many laboratories rely on other immunosystems for the diagnosis of dengue infections: ELISAs, immunochromatographic assays, or dot-blot assays (Vaughn, Nisalak et al. 1998) (Vaughn, Nisalak et al. 1999) (Cuzzubbo, Vaughn et al. 2000). The most widely used are an IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) or a combination of IgM-capture and IgG-capture ELISAs. The interpretation of serological test results may be complicated by the occurrence of cross-reactive antibodies to antigenic determinants shared by other members of the flavivirus family (i.e. Japanese encephalitis virus). Commercial kits for the detection of IgG as well as IgM antibodies have become available and seem to be

useful and reliable for serodiagnosis of dengue virus infection but more evaluation is required (Report of the Scientific Working Group on Dengue, 2006. TDR/SWG/08).

### **1.2.5. Management of Dengue Infection**

Treatment for DF is supportive. Patients with DF may be treated as out-patients (Chin, Kang et al. 1993).

The major pathophysiologic changes that determine disease severity seen in DHF include leakage of plasma with resultant hypovolemia/shock and abnormal hemostasis that may cause severe bleeding. These changes occur before the fever falls and before onset of shock. The duration of plasma leakage is 24 to 48 h in most cases. Therefore, therapy in DHF is directed at replacement of lost plasma and maintenance of adequate circulatory volume for the 24 to 48 hours period of increased vascular permeability and management is essentially supportive and symptomatic. The prognosis depends mainly on the early recognition and treatment of shock. The key to success is frequent monitoring and changing strategies. Patients with DHF grade I and II should be closely monitored for signs of shock. The management of DSS (grade III and IV) is a medical emergency needing prompt and adequate fluid replacement for the rapid and massive plasma losses through increased capillary permeability. Although volume replacement is recognized as the critical therapeutic intervention, World Health Organization management guidelines remain empirical rather than evidence-based. Initial resuscitation with Ringer's lactate is indicated for children with moderately severe dengue shock syndrome. Colloidal fluids are indicated in patients with massive

plasma leakage and in whom a large volume of crystalloids has been given. Dextran 70 and 6 percent hydroxyethyl starch perform similarly in children with severe shock, but given the adverse reactions associated with the use of dextran, starch may be preferable for this group (Wills, Nguyen et al. 2005). The best regiment of fluid resuscitation in DSS is still controversial and further large-scale studies, stratified for admission pulse pressure, are indicated (Dung, Day et al. 1999; Ngo, Cao et al. 2001).

Frequent recording of vital signs and determinations of the patient's platelet count and hematocrit level are important in evaluating the results of treatment. Therefore, the haematocrite centrifuge is needed bedside in Intensive Care Unit. Apart from correction of electrolyte and metabolic disturbances, oxygen is mandatory in all patients of shock. Some patients develop DIC and need supportive therapy with blood products (blood, FFP and platelet transfusions). Pleural effusion and ascitis are common in cases of DSS, and if possible, drainage should be avoided as it can lead to severe hemorrhages and sudden circulatory collapse.

There is insufficient evidence to justify the use of corticosteroids in managing dengue shock syndrome. There were 2 prospective, randomized, controlled trials with small samples sizes (97 and 63) showed that a single dose of corticosteroids (hydrocortisone hemisuccinate, 50 mg/kg in one trial, methylprednisolone (30 mg/kg) in other) does not reduce mortality in DSS which does not respond to conventional critical care (Sumarmo, Talogo et al. 1982) (Tassniyom, Vasanawathana et al. 1993). As corticosteroids can potentially do harm, clinicians should not use them unless they are participating in a randomized controlled trial



comparing corticosteroids with placebo (Panpanich, Sornchai et al. 2006) and further investigation on the use of corticosteroids in DHF/DSS is needed.

### **1.3. Pathogenesis of Dengue Infection**

Major manifestations of DHF include (i) plasma leakage through elevated vascular permeability, (ii) hemorrhage, and (iii) thrombocytopenia. Dengue shock syndrome (DSS) is a potentially lethal complication of dengue virus infection associated with hypotension and leakage of plasma fluid into the extravascular space.

The pathogenesis of DHF and DSS is still controversial. Two main factors, which are frequently cited to explain the pathogenesis changes that occur in DHF and DSS, are virus factors and host factors.

#### **1.3.1 Virus factors**

The dengue virus belongs to genus *Flavivirus* (family *Flaviviridae*) that includes about 70 distinct viruses, all of which are serologically related and in majority of cases, maintained in nature by transmission from hematophagous arthropod vectors (mosquito or ticks) to vertebrate hosts. More than 50% of the flaviviruses have been associated with human diseases, and of these, the most important in terms of disease incidence are dengue virus, yellow fever virus, Japanese encephalitis virus, and tick borne encephalitis virus. Dengue virus is present in tropical and subtropical areas around the world, as these provide the ecological conditions required for maintaining the natural cycles of the virus. There are four antigenically-related serotypes, dengue virus types 1, 2, 3, and 4. (Monath 1996).

Dengue virus is a single stranded, positive sense RNA virus with 11 kb unfragmented genome surrounded by a lipid bilayer envelope. The uninterrupted open reading frame codes for three structural proteins and seven non-structural proteins. Three structural proteins are C (nucleocapsid), M (membrane associated protein) and E (envelope protein) and the seven structural proteins have been named as NS1, NS2, NS3, NS4, NS5, NS6 and NS7 (Lindenbach 2001). Dengue virion has a spherical shape with 40–50 nm in diameter. The nucleocapsid is about 30 nm in diameter and covered by envelope. The envelope is a lipid bilayer containing two envelope-associated proteins: the E and M proteins. The E protein is glycosylated and responsible for attachment to the cellular receptors and fusion with endosomal membranes. The E protein contains the main epitopes recognized by neutralizing antibodies. These epitopes can be dengue virus-specific or cross-reactive with other flaviviruses. It is suggested that neutralizing antibody plays a key role in protective immunity against dengue viruses. It is generally believed, but not proven, that infection with one DENV serotype provides lifelong immunity to that serotype of virus, but limited or negligible cross-protective immunity to the other serotypes (Monath 2002).

The pathology of DHF and DSS has been well studied (Bhamarapravati, Tuchinda et al. 1967) (Bhamarapravati 1989). Gross and microscopic pathologic studies of tissues taken at autopsy in Thailand have shown diffuse petechial haemorrhages of most organs, as well as serous effusions in the pericardial, pleural, and peritoneal cavities. Microscopically, perivascular edema and loss of integrity of endothelial junctions are found.

Dengue antigen can be demonstrated in endothelial cells, but there is no apparent damage to the blood vessels or endothelial cells. Dengue antigen has been detected in Kuffer cells, alveolar macrophages, mononuclear phagocytes in the skin, and circulating monocytes (Bhamarapravati 1997). The viruses have been isolated or detected in organs such as lymphoid organs, liver, spleen, kidney, and brain (Guzman, Alvarez et al. 1999). There is increased proliferation of reticuloendothelial cells in the bone marrow, spleen, lymph nodes, and lungs. Dendritic cells could be the early, primary targets of dengue viruses in natural infection and could play an important part in the pathogenesis of dengue infection through an increase in virus load and cell activation (Wu, Grouard-Vogel et al. 2000) (Libraty, Pichyangkul et al. 2001).

It has been assumed that virulence of the virus contributes to the development of DHF, virulent dengue virus strains cause DHF while avirulent dengue virus strains cause DF. This hypothesis assumes that dengue viruses, like all animal viruses, vary and change genetically as a result of selection pressures as they replicate in humans and/or mosquitoes and that there are some virus strains that have greater epidemic potential. Phenotypic expression of genetic changes in the virus genome may include increased virus replication and viremia, severity of disease (virulence), and epidemic potential (Gubler, Reed et al. 1978). There has been some evidence that support for this theory. Two important genotypes for dengue 2 virus have been identified, one, from southeast Asian origin, related to most of the DHF epidemics in southeast Asia and the Americas; and the other, the American genotype, only related to DF epidemics in the American region (Leitmeyer,

Vaughn et al. 1999) (Guzman, Deubel et al. 1995) (Watts, Porter et al. 1999). The 1981 Cuban epidemic has been considered one of the most severe DHF epidemics to date in the Americas (Kouri, Guzman et al. 1989). The genomic study of Cuban dengue 2 strains and the RNA from a liver sample of a fatal case demonstrated their similarity with southeast Asian strains, at least at the studied fragment (Guzman, Deubel et al. 1995) (Sariol, Pelegrino et al. 1999). Thus, the introduction of the Southeast Asian genotype coincided with the appearance of DHF in different countries in the Americas, while the original American genotype was only associated with DF, but not with DHF (Rico-Hesse, Harrison et al. 1997) (Rico-Hesse, Harrison et al. 1998).

A similar epidemiological situation to that in Cuba was reported in Iquitos, Peru. A dengue 1 epidemic occurred in 1990 followed 5 years later by a dengue 2 epidemic with DF; no DHF cases were observed in Peru. The dengue 1 viruses that affected Cuba in 1977 and Peru in 1990 were similar; but, the 1981 Cuban dengue 2 strain belonged to an Asian genotype and the Peruvian dengue 2 virus belonged to the American genotype (Watts, Porter et al. 1999). These results suggest that the Asian genotype possess one or more virulence determinants that are absent from viruses originating in the Americas.

The relation of specific genotypes with severe disease has also being observed for dengue 3 virus (Figueroa and Ramos 2000) (Briseno-Garcia, Gomez-Dantes et al. 1996).

Classically, the strains that were isolated from patients with DHF or DSS are often called “virulent strains”. However, their virulence cannot be confirmed because of

a lack of adequate animal models of disease. It has not been determined whether some strains are virulent because they grow well in vivo with or without enhancing antibodies, because they are highly cytopathic, or because they induce high levels of cytokines. Further studies are needed to elucidate the molecular basis underlying these possible different phenotypes.

It has been reported that viral titers in peripheral blood are higher in patients who develop DHF than in those with DF (Vaughn, Green et al. 2000). However, it is not determined why and how higher levels of viremia lead to unique clinical manifestations: plasma leakage, hemorrhagic manifestations seen in DHF. Recently, Libraty et al showed that maximum plasma viremia levels of DEN-3 correlated with the degree of plasma leakage and thrombocytopenia (Libraty, Endy et al. 2002), although the level of correlation was not particularly strong.

### **1.3.2 Host factors and Immunopathogenesis**

A well-established epidemiologic feature of DHF /DSS is age. In the Cuban DHF /DSS outbreak of 1981, where everyone from 2 to 40 years of age experienced the same sequence of exposure (DEN-1 in 1977-78 and DEN-2 in 1981), hospitalized patients peaked at age 8 to 11 years and then fell dramatically nearly to the baseline among mid-teens (Kouri, Guzman et al. 1989). In addition, children are in higher risk of DHF than adults, as have been reported in Thailand and some others Southeast Asian countries. These studies showed that age-specific DHF incidence was bimodal, with severe cases peaking at 7 months of age and again at 3–5 years of age (Nimmannitya 1987). (Halstead 1970) (Guzman, Kouri et al. 1984). It has been suggested that baseline microvascular permeability of capillaries in children

is considerably greater than that of adults and this could explain, at least partly, why dengue shock syndrome is more frequently observed in children (Bethell, Gamble et al. 2001). Age associated immune status of the population can also govern the severity of outbreaks. When a single dengue serotype has been endemic for a generation or more, most adults will be solidly immune. A highly immune adult population and a semi-immune population of children would be expected to create an epidemiologic situation, in which there is little or no reported disease of any kind. This is what was noted during the era of European colonization of tropical Asia; dengue fever attacked the newcomers, but without obvious disease among the local population (Halstead 1994).

The interactions between pre-existing pathology and dengue infections are also contribute to the severity of illness. The best documented example is the severe gastrointestinal bleeding which accompanies dengue infections (primary or secondary) in individuals with peptic ulcer disease (Tsai, Kuo et al. 1991). Severe menorrhagia has also been described in women during dengue infections (Tai, Chee et al. 1999) (McGready, Paw et al. 2000). Individuals with open mucosal lesions, whether pathologic (peptic ulcer) or physiologic (menstruation), who suffer dengue infections may bleed. Blood loss may be catastrophic and life threatening.

Host immunopathogenesis is an alternatively hypothesis for the pathogenesis of dengue infection. This hypothesis implies that patients experiencing a second infection with a heterologous dengue virus serotype have a significantly higher risk for developing DHF and DSS (Halstead 1970) (Halstead 1988).

Antibody levels may have a dominant role in driving dengue infection to more or fewer infected cells. Epitopes present on E protein are able to induce neutralizing antibodies, both homologous and heterologous. People infected with one serotype maintain a life-long protective immunity to infection by the homologous virus, although protective immunity to infection with heterologous serotypes is short-lived. Cross-reactive antibodies that lack neutralizing activity are also induced in the primary infection. Upon secondary infection with a heterotypic virus, these preexisting heterologous dengue antibodies recognizes the infecting virus and forms virus-antibody complexes, which then bind to Fc $\gamma$  receptors on target cells, especially macrophages. Because the antibody is heterologous, however, the virus is not neutralized, and the non-neutralizing, cross-reactive antibodies markedly augment dengue virus infection of Fc $\gamma$  receptor-positive cells (Mady, Erbe et al. 1991). This phenomenon is called antibody-dependent enhancement (ADE). Thus, it is hypothesized that prior infection, through a process known as ADE, enhances the infection and replication of dengue virus in cells of the mononuclear cell lineage (Halstead and O'Rourke 1977) (Halstead and O'Rourke 1977) (Brandt, McCown et al. 1982) (Halstead 1988). It is thought that these cells produce and secrete vasoactive mediators in response to dengue infection, which causes increased vascular permeability leading to hypovolemia and shock. Infants born to the mothers develop DHF in the primary infections. The levels of maternal dengue virus antibodies in the infants needed to decline to the levels that can enhance dengue virus infection and lead to DHF (Kliks, Nisalak et al. 1989) (Kliks, Nimmanitya et al. 1988). These observations are consistent with the idea that

enhancing antibodies increase the number of dengue virus-infected cells and the levels of viremia and lead to DHF.

Activation of complement is another important clinical manifestation in DHF and thus it has been assumed that complement activation is also responsible for the pathogenesis of DHF. It was reported that the complement system is activated in DHF/DSS (Ward 1973) (Nishioka 1974). The peak of activation and the presence of C3a and C5a coincided with the onset of shock and leakage. The levels of C3a correlated well with disease severity (Malasit 1987). The mechanism of complement activation in DHF is not completely understood. Circulating immune complexes are present in the DHF patients, and it has been assumed that the complement is activated by the immune complexes (Malasit 1987). High levels of secreted NS1 and pre-existing cross-reactive antibody may also mediate complement activation (Avirutnan, Punyadee et al. 2006). Further, it was reported that dengue virus-infected monocytes and endothelial cells activate complement via classical and alternative pathways (Avirutnan, Malasit et al. 1998). It is likely that complement is activated by various mechanisms in DHF patients, and that complement activation also contributes to the pathogenesis of various clinical features, especially plasma leakage.

The cellular immune response may also be important in the pathogenesis of dengue. Circulating dengue virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were detected in individuals after a primary dengue virus infection (Bukowski, Kurane et al. 1989) (Kurane, Innis et al. 1989). It has been demonstrated that memory dengue T lymphocyte response after a primary infection includes both serotype-



specific and serotype-cross-reactive CD4+CD8(-)T lymphocytes and CD4(-)CD8+T lymphocytes. The predominant response in PBMC was to the serotype of dengue virus with which the donors had been infected, but cross-reactive responses to one or more of the other (heterologous) serotypes of dengue virus were detected in all subjects. (Kurane, Innis et al. 1990). Heterogeneous dengue virus-specific CD4+ cytotoxic T cells are stimulated in response to infection with a dengue virus and that a nonstructural protein, NS3, contains multiple dominant T-cell epitopes (Kurane, Brinton et al. 1991). NS3 protein seems to be the major target for CD4+ and CD8+ T cells, although some T cell epitopes have been recognized in other proteins such as envelope and capsid (Kurane, Zeng et al. 1998) (Spaulding, Kurane et al. 1999).

The pattern and magnitude of the cross-reactive response to heterologous dengue serotypes is variable between subjects, even among those who were infected with an identical virus (Dharakul, Kurane et al. 1994), depending on different factors such as the serotype causing the primary infection and the ethnicity of the individual (Rothman and Ennis 1999). Both CD4+ and CD8+ dengue virus-specific T lymphocyte clones are able to lyse dengue virus-infected target cells in vitro (Gagnon, Zeng et al. 1996) (Kurane, Innis et al. 1989) (Livingston, Kurane et al. 1995).

A series of studies have suggested that plasma leakage is caused by malfunction of vascular endothelial cells induced by cytokines or chemical mediators rather than by destruction of the small vessels and cytokines that may induce plasma leakage such as interferon  $\gamma$ , interleukin (IL) 2, and tumour necrosis factor (TNF)  $\alpha$  are

increased in DHF cases (Kurane and Ennis 1992) (Bethell, Flobbe et al. 1998) (Hober, Nguyen et al. 1998) (Rothman and Ennis 1999).

Cytokines could be released either directly from monocytes/macrophages as a result of infection or after interactions between infected and immune cells, or both (Kurane and Ennis 1992) (Hober, Nguyen et al. 1998). CD4<sup>+</sup> T lymphocytes produce a number of cytokines, including interferon  $\gamma$  (IFN $\gamma$ ), interleukin (IL) 2, IL-4, IL-5, IL-6, IL-10, and lymphotoxin. Moreover, monocytes/macrophages which are infected by dengue viruses produce tumour necrosis factor (TNF)  $\alpha$ , IL-1, IL-1B, IL-6, and platelet-activating factor (PAF). Finally, cytokine and chemical mediator production is induced by other cytokines. Thus, once cytokines are produced, a complex network of induction may further increase the levels of cytokines and chemical mediators, resulting in even higher levels with synergistic effects on vascular permeability (Kurane 1997). Interferon  $\gamma$  enhances uptake of dengue particles by target cells through increasing Fc cell receptors (Kontny, Kurane et al. 1988). Others cytokines such as IL-6, IL-8, and IL-10 are also increased (Green, Vaughn et al. 1999).

It has been, however, difficult to attribute the pathogenesis of DHF to a sole cytokine. It is more likely that the multiple cytokines function in a synergistic and additive fashion, and induce the pathology and the clinical manifestations of DHF. Further studies are still needed for a better understanding of the precise contribution of cytokines to dengue pathology.

It has been demonstrated that dengue infected peripheral blood monocytes in ADE conditions generate soluble mediators that activate endothelial cells through the

enhanced expression of adhesion molecules such as VCAM-1 and ICAM-1 (Anderson, Wang et al. 1997). At the same time, high levels of TNF $\alpha$  (induced by infected monocytes and by T-cell activation or both) could be responsible in part for transient vascular damage. The role of TNF $\alpha$  in the pathogenesis of the disease is critical, and it probably initiates several processes relating to plasma leakage and haemorrhage (Anderson, Wang et al. 1997). Avirutnan and colleagues have shown that infection of human endothelial cells with dengue virus induces the secretion of RANTES and IL-8, and in the presence of anti-dengue Abs, the formation of nonlytic complement complexes. Furthermore, dengue virus infection of endothelial cells in vitro caused apoptosis. Complement activation, chemokine induction, and apoptotic cell death may act in concert to cause the fulminant but short-lived vascular leakage that is characteristic of DHF/DSS (Avirutnan, Malasit et al. 1998).

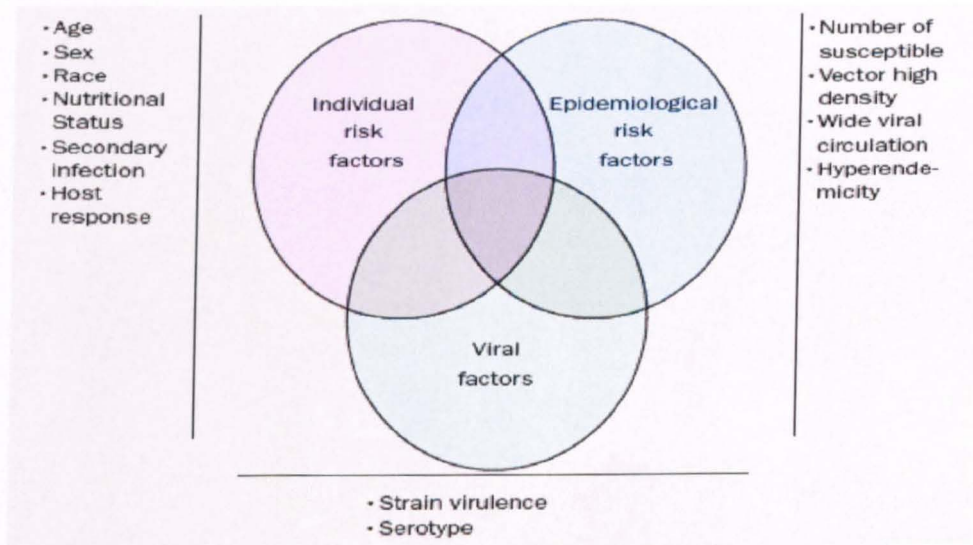
The potential role of mast cells/basophils in the pathogenesis of the disease also has been reported. Mast cell/basophile KU812 cells are permissive to dengue virus infection with the production of viral particles and vasoactive cytokine production.(King, Marshall et al. 2000).

### **1.3.3 Integrated hypothesis to explain pathogenesis**

Overall, there are many epidemiologic and laboratory-derived data to support important roles for both virus factors and host response to the overall clinical phenotype. These two theories have been considered as opposing each other for a long period of time. However, it appears that they are not mutually exclusive, and both are likely to be valid. Excellent reviews have recently been published on both

viral pathogenesis and immunopathogenesis (Kurane 1997) (Rothman and Ennis 1999) (Guzman and Kouri 2002). In essence the overall clinical outcome likely depends on a very complex interaction between virus, host, and host immune response. An integrated hypothesis for the development of DHF epidemics was proposed in 1987 (Kouri, Guzman et al. 1987). The intersection of three groups of factors (host, viral, and epidemiological factors) determine the nature of a DHF epidemic (Fig 1.4).

Figure 1.5: Risk factor for DHF/DSS: an integral hypothesis



(From Guzman et al. *Lancet Infectious Diseases* 2002) (Guzman and Kouri 2002)

In general, the epidemiological and viral factors are the determinants for an epidemic of disease. Individual risk factors such as age, chronic diseases are predisposing factors that make the disease more frequent in a certain age group (Nimmannitya 1987). (Halstead 1970) (Guzman, Kouri et al. 1984). However, the

pre-existence of immune response is the main individual risk factor, but not the only one, for the occurrence of severe disease. Individual risk factors are the ones that determine the appearance of the disease in a particular person in a given population. The presence or absence of these individual risk factors, in the matrix of epidemiological and viral factors, determines whether or not all persons with a secondary type of infection present the clinical picture of DHF.

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# **CHAPTER 2**

## **MATERIALS AND METHODS**

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## **2.1 Study site**

The Hospital for Tropical Diseases (HTD) (former name: Cho Quan Hospital) in Ho Chi Minh City is referral hospital for patients with infectious diseases, and serves the Ho Chi Minh City area (8 million population) and southern provinces of Viet Nam (population approximately 35 million). The hospital has 500 beds, including two intensive care units (paediatric and adult) and nine wards for non-complicated infectious diseases (malaria, DHF, diphtheria, HIV/AIDS, viral hepatitis, central nervous infections (encephalitis and meningitis), respiratory infections, acute diarrhea. The hospital staff have places in The National Committee of DHF Control, AIDS control, Malaria Control, Hospital infection Control and are collaborating with WHO on several aspects of dengue clinical management and diagnosis. Laboratories of HTD are supported by the Oxford University Clinical Research Unit to provide routine clinical haematology, biochemistry, microbiology, serology, immunology and molecular biology investigations. The HTD Laboratory is also involved in the TDR/PDVI network of laboratories for dengue diagnostic evaluations.

The Oxford University Clinical Research Unit (OUCRU) opened in 1991, funded by the Wellcome Trust of Great Britain. The OUCRU, located within the HTD, serves as a collaborative centre between HTD and Oxford University and started as an 8-bed ward for the treatment of patients with severe malaria. Over 10 years the Unit has expanded and now performs research on many areas including dengue, influenza, central nervous system infection.

## **2.2 Objectives of this thesis**

### ***Primary objectives***

To explore the factors those contribute to the pathogenesis of dengue infection in Vietnamese adults.

### ***Specific objectives:***

1/ to describe clinical features and immunological responses of adult patients with dengue infection admitted to the Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam.

2/ to investigate dengue-specific and cross – reactivity T Lymphocyte responses during acute disease and convalescence, in a population of naturally immune individuals, in a dengue-endemic region.

3/ to study the specificity and cross reactivity of short-term CD8<sup>+</sup> CTL lines and clones stimulated with different variants of the identified epitope from acute and convalescence PBMCs of secondary DHF patients. The functional phenotype of these cross-reactivity CD8<sup>+</sup> CTL lines and clones are also evaluated.

## **2.3 Patient and Study procedure**

Adult patients who admitted to the Hospital for Tropical Diseases with suspected dengue were recruited to prospective clinical descriptive and immunology studies. HTD Scientific and Ethical Committee approved all study protocols, and informed consent was obtained from each patient or accompanying relative.

### **2.3.1 Patient recruitment**

Consecutive adult cases ( $\geq 15$  year-old) presenting to the Hospital for Tropical Diseases in Ho Chi Minh city, Viet Nam who were suspected of having dengue



were invited to participate in the study between 2002 and 2004 and informed consent was obtained.

The case definition of a patient with suspected dengue included acute fever or history of acute fever (less than 7 days) with one or more of the following symptoms and signs: headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic phenomena, including a positive tourniquet test; exclusion of alternative diagnoses, such as malaria, by clinical examination and appropriate laboratory tests.

The World Health Organization (WHO) definitions and criteria were used to classify dengue disease ((WHO) 1997). DSS was defined as a case of DHF with either a narrow pulse pressure ( $\leq 20$  mm Hg) or unrecordable blood pressure ((WHO) 1986); typically these were accompanied by signs of circulatory insufficiency, such as cold extremities and thready pulse.

In parallel to the DHF/DSS classification, severe clinical manifestations of dengue were defined as internal hemorrhage, plasma leakage, shock, and/or platelet count  $< 50,000/\text{mm}^3$ . Internal hemorrhage consisted of melena, hematemesis, hematuria, and/or menorrhagia. Plasma leakage was indicated by the presence of pleural effusion, ascites, and/or haemoconcentration (haematocrit values  $\geq 20\%$  of the baseline value). Because baseline value was not known for most patients and only a small percentage of patients came back for follow-up to provide autologous baseline Hct values, haemoconcentration in adults for practical reasons was defined as haematocrit  $\geq 45\%$  (normal value of Hct in adult in Viet Nam is 38%, range from 35 – 41% (source: Ministry of Health of Viet Nam)).

The confirmation of acute dengue virus infection by elevated dengue virus-specific serum IgM titers as measured by capture ELISA (using a commercial PANBIO Dengue duo IgM and IgG test) and / or virology (using RT-PCR) (see 2.4).

### **2.3.2 Clinical procedure**

On admission to the study, following informed consent, patients were examined and results documented on a standardized Case Record Form (CRF) that also recorded the subject's demographic and epidemiologic characteristics as well as information regarding their illness. Clinical evolution of hospitalized patients was monitored daily using these CRF.

Venous blood was taken on admission for testing full blood count, blood glucose, serum creatinin, blood urea nitrogen and transaminases in the hospital laboratory, and serum was taken for serologic and virological testing. Patients were reassessed every 6 hours daily for vital signs as axillary temperature, pulse, blood pressure during febrile period until discharge. As per routine hospital procedure, blood samples were collected daily from patients hospitalized for dengue for monitoring of hematocrit and platelet levels until normal levels were obtained post-defervescence.

An extra sample for cytokine measurement was collected from consented patient as soon as possible after admission onto EDTA (1 mg/mL) and separated promptly, and plasma was stored at -70oC. A second blood sample, taken upon hospital discharge, served as the convalescent phase sample.

For cellular immune response studies, peripheral blood samples were collected from venous blood of patients between 8 and 11 am on the first morning after admission (study day 1), and again on study day 5 unless the patient was discharged earlier. Convalescent samples were taken at 14 days and 1 month after admission. Peripheral blood mononuclear cells (PBMCs) then were separated using Lymphoprep (Axis-Shield Poc AS, Oslo, Norway). Separated PBMCs were cryo-preserved in liquid nitrogen container for future immune assays.

Supportive therapy for all patients was given following the WHO guidelines and Viet Nam National Guideline for Dengue Hemorrhagic Fever. All patients who developed dengue shock syndrome were kept under close observation, including hourly measurements of pulse and blood pressure, hematocrit measurements every 2 to 6 hours with the bedside centrifuge by experienced physicians and nurses in intensive care unit. Fluid resuscitation was carried out immediately for these patients until they were no longer clinically hypovolemic. Fresh whole blood with matched group was given in the case of suspected internal bleeding whose had persistent shock even after initial resuscitation with plasma expanders and continuing decline of the haematocrit. Other appropriate treatments such as inotropic agents, mechanical ventilator were also applied if it is indicated. Usually, patients who had been resuscitated from shock rapidly recovered. Patients with dengue hemorrhagic fever discharged from the hospital after their clinical condition was stable, or at least 48 hours since recovery from shock in dengue shock syndrome. All patients were asked to come back to the hospital for follow up 2-4 weeks after discharge from hospital.

### **2.3.3 Routine Clinical Laboratory investigation**

Full blood counts were performed every day during hospitalisation. Blood glucose, creatinine were tested on admission and re-tested until the results were normal or the patient fully recovered from shock status. Chest X ray and ultrasound were performed for indicated severe cases. All routine clinical laboratory tests were performed followed standard operating procedures at the hospital laboratory.

## **2.4 Laboratory Methods**

### **2.4.1 Serology confirmation for acute dengue infection**

Dengue virus infectious cases were confirmed via serological testing of acute and early convalescent phase plasma with a commercial capture-immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) (Panbio, Brisbane, Australia). The ELISA was performed and results were interpreted according to the manufacturer's instructions. Results were determined by comparison with IgM and IgG reference sera provided (cut-off calibrators). A positive sample was defined as having a calibrator absorbance ratio  $\geq 1.0$  and a negative sample with a ratio  $\leq 1.0$ . Dengue virus infection was characterized by the elevation of either IgM or IgG, with a negative sample being defined as having both IgM and IgG ratios of  $<1.0$ . The Panbio ELISA has been calibrated against HAI units previously (Vaughn, Nisalak et al. 1999); an IgG level greater than 40 units has been defined as highly suggestive of secondary dengue. This PANBIO Dengue duo capture-IgM and IgG ELISA assay has been proven to be a reliable, rapid, sensitive, and specific diagnostic test to diagnose primary and secondary dengue infection on

single or paired samples (Cuzzubbo, Vaughn et al. 1999) (Vaughn, Nisalak et al. 1999).

## **2.4.2 Dengue virus molecular serotyping methods**

### **2.4.2.1 RNA Extraction**

Dengue RNA was extracted from plasma using the Boom method (Boom, Sol et al. 1990).

#### **Preparation of reagents:**

- Buffer L6: add 120 g of guanidinium isothiocyanate to 100 mL of 0.1 M Tris/HCl pH 6.4 and 22 mL of 0.2 M EDTA pH 8.0, and 2.6 g of Triton X-100; stir overnight in the dark to dissolve. Store in the dark.
- Buffer L2: add 120 g of guanidinium isothiocyanate to 100 mL of 0.1 M Tris/HCl pH 6.4 and stir overnight in the dark to dissolve. Store in the dark.
- Size fractionated silica, add 60 g of silicon dioxide to 500 mL of distilled water in a measuring cylinder and allow standing for 24 h at room temperature. Discard 430 mL of supernatant and re-suspend solids in 500 mL of distilled water. Allow to stand at room temperature for 5 h and discard 440 mL of supernatant. Add 600  $\mu$ L of concentrated HCl pH 2.0 and aliquot into 1.5 mL volumes. Sterilize by autoclaving (15lbs for 15min) and store in the dark.

**Protocol:** 50-200 $\mu$ L of serum, plasma or culture supernatant was added to a tube containing 1 mL of L6 lysis buffer and 20 $\mu$ L of a suspension of size-fractionated silica particles, then briefly vortexed. After incubation for 10 min at RT, the tube was vortexed for 5 seconds and centrifuged for 1 min at 13,000 x g. Supernatant

was then discarded and 1 ml of washing buffer L2 added to the silica-nucleic acid complex, followed by vortexing (5 sec) and two washes with buffer L2 (1 min at 13,000 x g), followed by two washes with 70% (vol/vol) ethanol and one wash with 1ml of acetone (Merck). After disposal of the acetone, the tube was dried at 56°C with open lid in a heating block for 10 min. The pellet was resuspended in 100µl of 1x TE buffer, vortexed for 5 seconds, and left at 56°C for 10 min to elute the nucleic acids. After incubation, it was vortexed again and centrifuged for 2 min at 13,000 x g. Only 70 µl of the supernatant containing viral RNA were gently pipetted to a new 0.5ml eppendorf to avoid disturbing the pellet. In most cases, RNA was immediately used for synthesis of cDNA, otherwise it was stored at minus 80oC until used.

#### **2.4.2.2 Oligonucleotide primers**

Dengue virus consensus primers D1 and D2 that were designed for maximum homology to the four serotypes, high melting temperature, and nonhomology to other regions of dengue virus genomes are shown in Table 2.1, along with their genome positions and product sizes when used in enzymatic amplifications. The type-specific primers shown in Table 2.1 (TS1, TS2, TS3, and TS4) were designed to anneal specifically to each of their respective genomes.

Table 2.1: Oligonucleotide primers for amplification and serotyping dengue virus

Primes	Sequence	Genome position	Size (in bp)
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	134-161	511
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616-644	511
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	568-586	482 (D1 and TS1)
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	232-252	119 (D1 and TS2)
TS3	5'-TAACATCATCATGAGACAGAGC-3'	400-421	290 (D1 and TS3)
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	506-527	392 (D1 and TS4)

#### 2.4.2.3 Lanciotti seminested RT-PCR

A seminested RT-PCR, targets the C and preM region has been developed (Lanciotti, Calisher et al. 1992). It uses universal outer dengue primers D1 and D2, followed by a subsequent serotype-specific seminested PCR combining primer D1 with one of the following internal primers: TS1, TS2, TS3, or TS4.

Target viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by use of reverse transcriptase (RT) and the dengue virus downstream consensus primer (D2), homologous to the genomic RNA of the four serotypes as following: RNA pellet was resuspended in 30  $\mu$ l of RNase-free water and incubated for 10 min at 55°C, and then 5  $\mu$ l was used in an RT reaction in a 15- $\mu$ l mixture containing 500  $\mu$ M deoxynucleoside triphosphate (dNTP), 0.25  $\mu$ M D2 primer, 20 U of RNasin (Promega), 5 U of avian myeloblastosis virus reverse transcriptase (Promega), 5 $\times$  RT buffer, and RNase-free water. RT was carried out at 42°C for 1 h.

The first-round PCR was carried out in a 50- $\mu$ l volume containing 2.5 mM MgCl<sub>2</sub>, 0.2 mM (each) dNTPs (Boehringer Mannheim, Mannheim, Germany), 0.2  $\mu$ M (each) D1 and D2 outer primers, 1 $\times$  Taq buffer, 2 U of Taq polymerase (Promega), 5  $\mu$ l of the RT product, and PCR-grade water. The PCR program started with 5 min of denaturation at 95°C, followed by 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and ended with a 72°C final extension step for 10 min. The second-round PCR was performed in a 50- $\mu$ l reaction mixture containing 2.5 mM MgCl<sub>2</sub>, 0.2 mM (each) dNTPs, 0.2  $\mu$ M (each) D1, TS1, TS2, TS3, and TS4 primers, 10 $\times$  Taq buffer, 2 U of Taq polymerase, and 5  $\mu$ l of the diluted first-round PCR product (1:100 in PCR-grade water). The cycling program started with a 5-min denaturation step at 94°C, followed by 25 cycles each at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and ended with a 72°C final extension step for 10 min. Positive (dengue viral RNA) and negative (no template) controls were included for each step of the process (extraction, RT, and first- and second-round PCR).

This assay is known to have the good sensitivity and is widely used for diagnosis and surveillance of dengue, particularly in Southeast Asian countries (Nguyen, Tran et al. 1997) (Solomon, Dung et al. 2000) (Vaughn, Green et al. 2000).

#### **2.4.3 Separation of peripheral blood mononuclear cells from peripheral blood.**

Peripheral blood mononuclear cells (PBMCs) were separated using Lymphoprep (Axis-Shield Poc AS, Oslo, Norway). Whole blood was placed on the lymphoprep (ratio 1:1 to 1:1.5) using a pipette and applying the sample gently to the sides of 50ml Falcon tube already containing the right amount of Ficoll. This tube was



centrifuged at 2000x g (Relative centrifugal force) for 20-25 minutes at room temperature with no brake. After centrifugation, the milky – white - cloudy layer (PBMC layer) at the interface between the Ficoll (transparent) and the remaining plasma (yellow) was transferred into other another 50ml tube by aspirating gently with a sterile pipette. PBMCs were washed twice with RPMI (normally 10-20 mL are used). PBMCs were re-suspended in R/10 (RPMI 1640 medium containing 10% HI-FCS, 2mM L-glutamine, 100µg/ml Streptomycin, 100U Penicillin) for direct use in immune assay or future use in cryo-preservation.

#### **2.4.4 Cryopreservation of PBMCs.**

PBMC were cryopreserved at a cell concentration of  $5-10 \times 10^6$  viable cells/mL in cold freezing medium (heat inactivated FCS + 10% Dimethylsulfoxide - DMSO) and aliquoted to cryovials (Corning Incorporated, CORNING, Mexico). The cryovials were immediately placed in a slow-freeze container (e.g. “Mr Frosty”) which was then placed in a -70°C freezer for 4 to 24 hours. After 4-24 hours, the cryovials were transferred into a liquid nitrogen tank (-135°C) for long-term storage.

#### **2.4.5 Thawing of cryopreserved PBMCs.**

Cryovials containing frozen PBMC were removed from liquid nitrogen storage and quickly defrost by immersion and gentle shaking in a 37°C water bath until the majority of the suspension had thawed. The suspension was then transferred to a 15mL centrifuge tube with sterile pastette. Immediately add warm (37°C) R/10 medium (RPMI 1640 medium containing 10% HI-FCS, 2mM L-glutamine, 100µg/ml Streptomycin, 100U Penicillin); initially slowly with gentle shaking, 0.5

mL/min for the first 2 minutes, followed by 2 mL/min for the next 2 minutes, and then top up with 5 mL. The thawed cells then were washed twice with RPMI. The supernatant was discarded; the pellet was flicked and re-suspended in cRPMI and ready for use.

#### **2.4.6 Establishing CTL lines and CTL clones**

Short-term CTL-cell lines are produced by direct stimulation PBMCs with epitope peptide. PBMCs were divided according to the number of peptides to be used and centrifuged at 600g for 5 minutes at room temperature in separate tubes. The pellet was resuspended in 100 $\mu$ L concentrated sterile peptide solution, to a final concentration of 20 - 50 $\mu$ M and incubated at 37°C, 5% CO<sup>2</sup> for 1-2 hours. The cells then were resuspended into 1-2 x 10<sup>6</sup>/mL in R/10 (RPMI 1640 medium containing 10% HI-FCS, 2mM L-glutamine, 100 $\mu$ g/ml Streptomycin, 100U Penicillin) and plated out in 24-well plates, i.e. 2-4 x 10<sup>5</sup>/well. IL2 was added on day 3 to a final concentration of 10%. After 6 – 10 days in culture, the cells were splitted if they look overcrowded, and maintained in R/10 containing IL2. Although some CTL activity can often be detected as early as day 7 – 10, specific killing is maximal after 12 – 14 days. The cultures were usually maintained specific CTL activity for up to 3-4 weeks without restimulation. Clones also were set up at this stage.

Epitope peptide specific Cytotoxic T Lymphocyte clones were generated by the limiting dilution of CTL cell lines. Briefly, CTL lines were plated out in flat – bottom 96-well microtitre plate at a concentration of 1 cell/well in cloning mixture containing irradiated mixed allogeneic PBMCs, irradiated autologous peptide –

pulsed BCLs, phytohaemagglutinin and 10% pooled human AB serum in RPMI (R/10) supplemented with 25 ng of IL-7/ml. On day 4, 50 U of recombinant IL2 per ml of R/10 was added. Wells demonstrating sufficient growth and cell density (very obvious on microscopy of flat-bottomed plate) at each 7-day period were screened for their specificity using tetramer staining and selected clones were expanded into 48-well (then 24-well) plates tissue culture plates with autologous, irradiated, peptide-pulsed EBV-transformed B-lymphoblastoid cells (B-LCL). The expanded clones were cultured for 14 days and were screened for their specificity using tetramer staining. Specific clones were maintained by weekly restimulation with autologous, irradiated, peptide-pulsed EBV-transformed B-lymphoblastoid cells (B-LCL) and also were kept frozen and stored in liquid nitrogen.

#### **2.4.7. PCR using Sequence-Specific Primers (PCR-SSP) for HLA typing**

PCR-SSP for HLA typing was kindly performed by Tim Rostron from Institute of Molecular Medicine - Oxford. Briefly, typing of HLA class I and class II alleles was performed using the amplification refractory mutation system PCR (ARMS-PCR) with sequence specific primers, as described previously (Bunce, O'Neill et al. 1995). One hundred and ninety two PCR reactions were performed to genotype HLA-A, B, Cw, DRB1 and DQB1 alleles PCR was performed using 96-well polycarbonate plates (Thermo-Fast 96, Low profile, ABgene, UK), and a Peltier thermal cycler machine (DNA Engine Tetrad 2, BIO-RAD). Each well of a 96-well PCR plate contained a unique set of allele-specific PCR-primers, and a set of internal control primers (amplifying a conserved region from the third intron of the HLA-DRB1 locus). The presence of an allele is defined as when the allele-specific

PCR product is present and negative when only the internal control product is present.

#### **2.4.8 Methods for measuring Cytotoxic T Lymphocyte Activity**

There are a number of different assays of CTL activity, which assess different aspects of their function. The full details of these methods are described in the following sections.

The best-known assay that looks at the killing efficiency of CTLs is so called CTL lysis assay. This conventional method aims to examine the lysis of appropriate target cells expressing the correct HLA restriction molecule and presenting the specific antigen. This chromium release assay has been mainstay of this approach to measuring CTL activity for over two decades. The target cells are labeled with radioactive  $^{51}\text{Cr}$  for 1 h before use, and then incubated with cultured CTLs in vitro. Specific CTL activity is calculated from the excess release of chromium in the presence of a specific antigen compared to that released from the target cells without CTL or without antigen. These assays are usually carried out using several dilutions of CTLs (i.e. varying the effector:target (E:T) ratio) or of antigen (e.g. peptide titration) in order to demonstrate that this is a titratable response. In addition, measuring those CTLs that are capable of expanding and growing over 2-3 weeks in culture, which are then assayed for antigen-specific cytotoxicity using CTL lysis assay is a principle of Limiting Dilution Analysis (LDA). LDA was the 'gold standard' assay for many years for quantitation of antigen-specific T cells. In other hand, most CTLs release a variety of cytokines and chemokines, most commonly IFN $\gamma$  and TNF $\alpha$ , on antigen-specific contact (Lin and Askonas 1981)

(Morris, Lin et al. 1982). It is therefore possible to use the antigen-specific release of IFN $\gamma$  or TNF $\alpha$  as an indication of CTL recognition. The Enzyme-Linked Immuno - Spot (ELISpot) method has been adapted to provide a simple and rapid assay for the identification and enumeration of CTLs, on the basis of their antigen-specific secretion of TNF $\alpha$  (Herr, Schneider et al. 1996) or IFN $\gamma$  (Lalvani, Brookes et al. 1997). The ELISpot assay detects cytokines produced by an individual cell, which are then captured by binding to a specific antibody immobilized on the base of a micro titre well. When the assay is developed using a biotinylated second-layer antibody, followed by the addition of streptavidin conjugated to alkaline phosphatase, the use of a chromogenic alkaline phosphatase substrate stains the area of cytokine release as a blue 'footprint' for each secreting cell. The spots in wells with and without antigen, and at different input cell numbers, are then counted by eye or using a dissecting microscope, and the results presented as the number of specific spot -forming cells (SFCs) per  $10^6$  PBMCs. The assays originally used a target-cell population to present the antigen, but when using sufficient numbers of PBMCs, the cells will present antigen to one another. The ELISpot assay has been most commonly used to screen for peptide-specific responses and to quantify these from PBMCs (Lalvani, Brookes et al. 1998) (Jameson, Cruz et al. 1998).

More recently, the use of peptide-HLA tetrameric complexes has provided a way of enumerating CTLs, based on the expression by CD8 $^+$  T cells of a T-cell receptor (TCR) capable forming a stable interaction with that particular HLA-peptide complex. Peptide-HLA tetrameric complexes allow the direct visualization

of antigen specific T cells by flow cytometry (Altman, Moss et al. 1996) (Ogg, Jin et al. 1998). Therefore, by itself, this method does not provide any information about the functional phenotype of the tetramer-staining population. However, the majority of sorted tetramer-positive cells will produce IFN $\gamma$  in an ELISpot assay (Dunbar, Ogg et al. 1998) (Murali-Krishna, Altman et al. 1998), and many of them will also expand as clones (Dunbar, Ogg et al. 1998).

These different methods have been directly compared in a study of EBV specific CTLs in 13 people chronically infected with EBV (Tan, Gudgeon et al. 1999). In terms of the hierarchy of epitopes recognized within an individual, each method gave similar results; however, the absolute frequency differed substantially according to the method used. There was good correlation between ELI Spot assays and both other methods.

The choice between these different methods will depend both on practical circumstances and on the aspect of CTL function under scrutiny. The CTL lysis assay remains a robust method of examining cytotoxic function, but requires the use of radioisotopes and usually necessitates the expansion of CTLs in culture over several weeks. The limiting dilution assay has been the 'gold standard' for enumerating antigen-specific CTLs for many years, but is a very time-consuming and exacting procedure, and appears to underestimate significantly the true frequency of circulating antigen-specific CTL effectors. The ELISpot assay is simple and quick, so it is a very useful procedure for screening potential CTL responses and new CTL epitopes. It also uses very few cells, so is valuable when samples are limited. However, once the assay is complete, the cells are lost and

cannot be manipulated any further. Moreover, only one secreted factor can be examined in each assay, so this provides only limited information about CTL phenotype. The tetramer assay has the advantage of providing phenotypic as well as quantitative information, since co-staining with other antibodies provides the opportunity to look at surface markers of activation, T-cell receptor usage, and cytokine and chemokine production (using intracytoplasmic staining). Moreover, the cells can be sorted directly from the FACS machine and expanded as CTL populations or individual clones (Tan, Gudgeon et al. 1999). However, this method does not provide directly any information about the functional phenotype of the tetramer-staining population.

#### **2.4.9 IFN $\gamma$ ELISPOT assay**

The IFN $\gamma$  ELISPOT assay was performed according to the manufacturer's instructions (Mabtech AB, Stockholm, Sweden). 96-well polyvinylidene difluoride (PVDF)-backed plates (MAIP, Millipore, USA) were coated with coating antibody 1-D1K (50ul at 15ul/ml), incubated at room temperature for 2 hours or overnight at 4°C. The capture antibody was discarded and the wells were then washed 6 times with 200ul of sterile phosphate-buffered saline (PBS) or RPMI. The blocking solution was applied at 200 $\mu$ l/well (R/10) for at least one hour at room temperature. PBMC samples ( $5 \times 10^4$  to  $1 \times 10^5$  cells/well) then were plated out in 100 $\mu$ L/well of serum-containing medium (R/10) and stimulated with solution of individual peptides or peptide pools at required concentration (usually individual peptides were used at 20 $\mu$ M final concentration, peptide pools were used at a final

concentration of 5 $\mu$ M for each peptide). PBMC were incubated overnight with peptides at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. The plate should not be disturbed at this stage of the assay. After overnight incubation, the cells and medium were discarded, the wells then were washed six times with PBS containing 0.05% Tween-20 (Sigma). The detection monoclonal antibody, 7-B6-1-biotin (Mabtech, Sweden - diluted 1:1000 in sterile - filtered PBS at 50 $\mu$ l per well) was added to each well for 2-4 hours at 37°C. The wells were washed again 6 times with PBS containing 0.05% Tween-20 before incubating with 50 $\mu$ l of conjugate antibody (Mabtech, Sweden - at a dilution of 1:1,000 in PBS) for one hour at 37°C. After washing again 6 times with PBS + 0.05% Tween-20, chromogen was added at 100 $\mu$ l per well. The plate was left at room temperature until the blue color appears. The reaction was terminated by washing the plate under running tap water. The number of spot-forming units (SFU) in each well was counted with the aid of a dissecting microscope, and the background (no antigen stimulation) was subtracted. For each assay, the phytohemagglutinin (Sigma, Poole, UK) was used as the positive control. At the time of this study, an automated ELISPOT reader was not available. Therefore, ELISPOT counting was done manually. This approach may have limitations in terms of observer bias.

#### **2.4.10 FLOW cytometry and MHC Class I Tetramer staining**

##### **2.4.10.1 FLOW cytometry**

Fluorochrome-conjugated monoclonal antibodies were used to stain whole blood, CTL lines and clones and all analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson). Absolute and percentage counts of leukocyte



subsets were obtained by flow cytometry using a FACSCalibur four-color flow cytometer (Becton Dickinson, San Diego, USA). The surface phenotype of T cells was determined using panels of mAb such as CD8<sup>+</sup>, CD38<sup>+</sup> and other tetrameric-peptide complexes. Briefly, 100ul of whole blood or 10ul of CTLs pellet were stained with each mAb cocktail at room temperature in the dark, fixed by Facs-lyse, and then washed twice by PBS. Cells were resuspended in 1% Para formaldehyde and analyzed by flow cytometry

#### **2.4.10.2 Tetramer staining**

Tetrameric HLA - A11 /peptide complexes (“tetramers”) were synthesized as previously described (Altman, Moss et al. 1996). Briefly, the HLA molecule heavy chain cDNAs were modified by substitution of the transmembrane and cytosolic regions with a sequence encoding the BirA biotinylation enzyme recognition site, These modified HLA heavy chains, and  $\beta$ 2-microglobulin, were synthesized in a prokaryotic expression system (pET, R&D Systems), purified from bacterial inclusion bodies, solubilised in the presence of denaturant (4M Urea) and allowed to refold with the relevant peptide by dilution. Refolded monomeric complexes were purified by FPLC and biotinylated using BirA enzyme (Avidity), then combined with either phycoerythrin (PE)-labeled streptavidin (Sigma) or Quantum red (Sigma) at a 4:1 molar ratio to form tetrameric HLA/peptide complexes (“tetramers”).

Cell surface staining was carried out on whole blood (200 $\mu$ l), freshly separated PBMCs, thawed cryo-preserved PBMCs or cell clones. Cells were incubated with conjugated peptide - MHC class I tetramers (phycoerythrin fluorophore conjugated

or quantum-red conjugated) for 30 min in the dark. Sample was stained with an APC-conjugated anti-CD8 antibody for 15 min at room temperature in the dark. Red cells (if whole blood staining) were then lysed by 1X lysing buffer (BD Immunocytometry Systems) for 10min at room temperature. Cells were then washed three times with PBS and stored in Cell Fix<sup>TM</sup> buffer (Becton Dickinson) at 4°C until flow cytometry analysis was performed. Samples were analyzed on a Becton Dickinson FACSCalibur.

#### **2.4.11 Cytotoxic T Lymphocyte lysis assay**

After 14 days of culture, cells were harvested and used as effectors in a <sup>51</sup>Cr-labeled release assay. Target cells in the chromium release assay consisted of a <sup>51</sup>Cr-labeled B-cell line (BCL) that was autologous or Class 1 HLA-matched with effectors. The BCL was pulsed for 1 hour with the epitope peptide, washed, and aliquoted in duplicate in 96-well round-bottomed microtitre plates ( $5 \times 10^3$  to  $1 \times 10^4$  cells/well). Then effector cells were added in a range of effector-to-target ratios (50-100:1 for short-term CTL line, lower ratio for clones such as 1:1). The control wells were plated out for background <sup>51</sup>Cr release (target cells plus 100μL R/10) and total <sup>51</sup>Cr release (target cells plus 100μL detergent – 5% Triton X100 in water) for each target in quadruplicate. Detergent was added last of all and avoiding splashing it into any of other wells. Nonspecific killing of un-pulsed target cells will be subtracted from that of pulsed target cells. The plates were incubated for 4-6 hours at 37°C in 5% CO<sub>2</sub>. 20μL of the supernatant of each well were carefully removed (without disturbing the cells) onto beta-plate and count in beta counter.

The  $^{51}\text{Cr}$  release was calculated from the following equation:

$$\%lysis = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}$$

These assays are carried out using several dilutions of CTLs (i.e. varying the effector:target (E:T) ratio) or of antigen (e.g. peptide titration) in order to demonstrate that this is a titratable responses.

#### **2.4.12 Cytokine assays**

##### **2.4.12.1 Cytokine measures by sandwich ELISA**

Cytokine assay were performed by sandwich ELISA in 96-well microtiter plates following the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for cytokine has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any cytokine present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for cytokine was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. After an incubation period, an amplifier solution was added to the wells and color was developed in proportion to the amount of cytokine bound in the initial step. The color development was stopped and the intensity of the color was measured. The average of the duplicate readings for each standard and sample were subtracted the average zero standard optical density. The optical density for the standards versus the concentration of the standards was plotted and the best

curve was drawn. The data was linearized by using log/log paper and regression analysis was applied to the log transformation.

The concentration of each mediator was estimated with reference to recombinant standards included on each plate. The detection limits were 10 pg/mL for TNF $\alpha$ , IL-6 and IL-10; 100 pg/mL for sTNFR-55 and sTNFR-75.

#### **2.4.12.2 Cytokine measurement by Cytometric Bead Array**

Cytokine and chemokine concentrations release from clones were measured using a Cytometric Bead Array (Becton Dickinson, San Diego, USA). The BD CBA system used the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a CBA provided a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA capture bead mixture was in suspension to allow for the detection of multiple analytes in a small volume sample. The broad dynamic range of fluorescence detection via flow cytometry required fewer sample dilutions and substantially less time overall (compared to conventional ELISA) to establish the value of an unknown. The BD Human Th1/Th2 Cytokine CBA Kit was used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-10 (IL-10), Tumor Necrosis Factor  $\alpha$  (TNF- $\alpha$ ) and Interferon- $\gamma$  (IFN- $\gamma$ ) protein levels in a single sample.

The experiment was followed the assay procedure which was recommended by manufacturer. A mix of distinct fluorescence intensities beads from brightest to dimmest and recombinant standards or test samples were incubated together and then the sandwich complexes were detected by PE – conjugated anti-human

antibodies. Standard curves were set in every experiment for cytokine/chemokine concentration analysis.

#### **2.4.13 Intracellular Cytokine Surface staining**

Staining for intracellular cytokines, followed by flow cytometry analysis, was provided single-cell information about a cell population that one cannot obtain from surface staining or ELISA. CTLs were directly stimulated with epitope peptide for cytokine producing. GolgiPlug™ (BDB-Pharmingen; containing brefeldin A) was used for blocking intracellular transport processes and results in the accumulation of most cytokine proteins in the rough endoplasmic reticulum or Golgi complex. These activated cells then were suspended and distributed to plastic tubes or microwell plates for immunofluorescent staining. Cell surface was stained with fluorochrome-conjugated monoclonal antibody specific for a cell surface antigen, such as, CD3, CD8, tetramer (15-30 min, 4°C). As some antibodies, which recognize native cell surface markers may not bind to fixed/denatured antigen, it is recommended that the staining of cell surface antigens be done with live, unfixed cells PRIOR to fixation/permeabilization and staining of intracellular cytokines. Cells were fixed by adding 100µl of 4% Formaldehyde and mixed well. The cells were washed twice with ice-cold PBS/BSA/Azide buffer and resuspended in 150 ml of permeabilization buffer (Perm/Wash™) for 15 min. Intracellular Cytokines were stained with respective fluorochrome-conjugated anti-cytokine antibody. The cells then were washed 2 times with 1X Perm/Wash™ solution (1 ml/wash for staining in tubes) and resuspended in Staining Buffer prior to flow cytometric analysis.

## **2.5 Statistical analysis**

Most of the data were not normally distributed and therefore nonparametric tests (Mann-Whitney U-test, Wilcoxon signed rank test for pairs) were used. Wilcoxon signed rank test for pairs was used to compare paired data from each individual (level of cytokine in acute and convalescent phase in same patients). The Chi-square test with Yate's correction or the Fisher's exact test was used to compare proportions. The software package SPSS was used for all analyses

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**CHAPTER 3**

**CLINICAL FEATURES OF**

**DENGUE INFECTION IN ADULTS**

**IN VIET NAM**

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### **3.1 Introduction**

During the past few years, the characteristics of dengue in Viet Nam appear to have changed. For instance, a decade ago, children were predominantly affected, but in recent years, clinicians have seen increasing numbers of adult dengue patients, with both significant morbidity and increasing numbers of adult deaths due to dengue.

The rise in dengue among Vietnamese adults adversely affects development of economy as it affects the most economically productive people in the population. It also affects health planning, and is further compounded by the general lack of systematically collected information on the natural history of dengue in such patients. This lack often leads health planners and clinicians to base their decisions regarding resource allocation and clinical management on personal experiences of dengue management in children, rather than on prospective evidence from this population.

This study, conducted at the Hospital for Tropical Diseases of Ho Chi Minh City in Viet Nam, was designed to recruit consecutively admitted adult patients with dengue in order to systematically describe the clinical features and immunological responses. The study was approved by the Scientific and Ethical Committee of the Hospital for Tropical Diseases.

Consecutive adult cases ( $\geq 15$  year-old) presenting to the Hospital for Tropical Diseases in Ho Chi Minh City, Viet Nam who were suspected of suffering from dengue underwent the informed consent process and were invited to participate in



the study between 2002 and 2004. Clinical procedures in the study were described in Chapter 2. Briefly, all information for study of patients was recorded in their separate CRF. Blood samples were taken for routine and research investigation. Supportive therapy for all patients were followed the WHO guideline and Viet Nam National guideline for Dengue Hemorrhagic Fever. All patients were asked to come back to the hospital for following up. Cytokine assay were performed by sandwich ELISA in 96-well microtiter plates, by use of previously described techniques (chapter 2). The concentration of TNF $\alpha$ , IL-6 and IL-10, sTNFR-55 and sTNFR-75 were measured with reference to recombinant standards included on each plate. Appropriate statistic analyses such as Wilcoxon signed rank tests for paired samples were performed using SPSS software.

## **3.2 Results**

### **3.2.1 Clinical findings**

Between June 2002 and April 2004, 182 patients with laboratory-confirmed dengue infections were analyzed among a total of 198 adults presenting to the HTD. Serologic responses could be defined as secondary in all 182 patients. There were 100 (55%) with dengue without shock and 82 (45%) with DSS. RT-PCR results were positive in 101 patients (55%), 71% of whom presented before the day 5 of the illness. DEN-1 was detected in 12 patients, DEN-2 in 65, DEN-3 in 6 and DEN-4 in 17 cases. There was one DHF grade II patient infected by serotypes 1 and 2. All 4 serotypes of dengue virus are responsible for all spectrum of clinical severity of dengue infection in this study (Table 3.1).

Table 3.1: Serotype Features of all patients

	DF/DHF I	DHF II	DHF III	DHF IV	Total
DEN-1	0	8*	4	0	12
DEN-2	3	31*	30	1	65
DEN-3	0	4	2	0	6
DEN-4	0	9	7	1	17

\*one DHF II patient was co-infected by DEN-1 and DEN-2.

### 3.2.1.1. Demographic characteristics

The male (n = 98) to female (84) ratio was 1.2:1. The mean age was 20 years (SD=5.5), the range of age from 15 to 52 years. The distribution of ages in the patient population is shown in Figure 3.1, note that the left skewed deviation of age with the peak of age at 17 years could be seen in this chart. Note that an adult is defined as >14 years of age.

Amongst the 182 patients, 148 patients (81%) lived in districts in Ho Chi Minh City, with the majority living in District 8, 6, 11, 5 and 4, an area south-west of the city and generally an area of lower socioeconomic development (Figure 3.2).

Together with 129 patients admitted directly to HTD, 19 patients were referred to HTD from another hospitals in Ho Chi Minh city and 34 patients who lived in other provinces were admitted to Provincial Hospital where they received treatment with an average hospital stay of 1 day (N= 53 SD=1.1, range: 0 – 4 days) until they were referred to the HTD. There were 28/53 patients (53%) had been received intravenous infusions with saline solution in provincial hospitals, three received colloid solutions and one had a blood transfusion.

Patients presented at the HTD after a mean 5 (SD= 1.2) days of illness and were hospitalized for a mean duration of 8 (SD= 2.4) days. The distribution of illness lengths prior to admission into HTD is showed in Figure 3.3.

Figure 3.1: Distribution of age

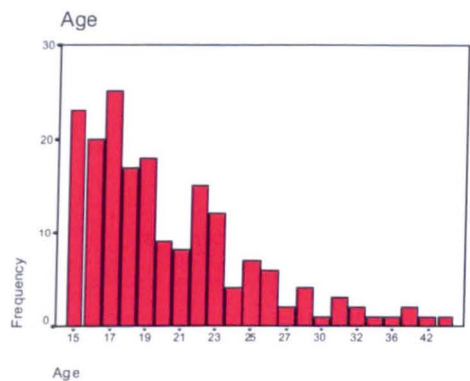


Figure 3.2: Location of patients in Ho Chi Minh City

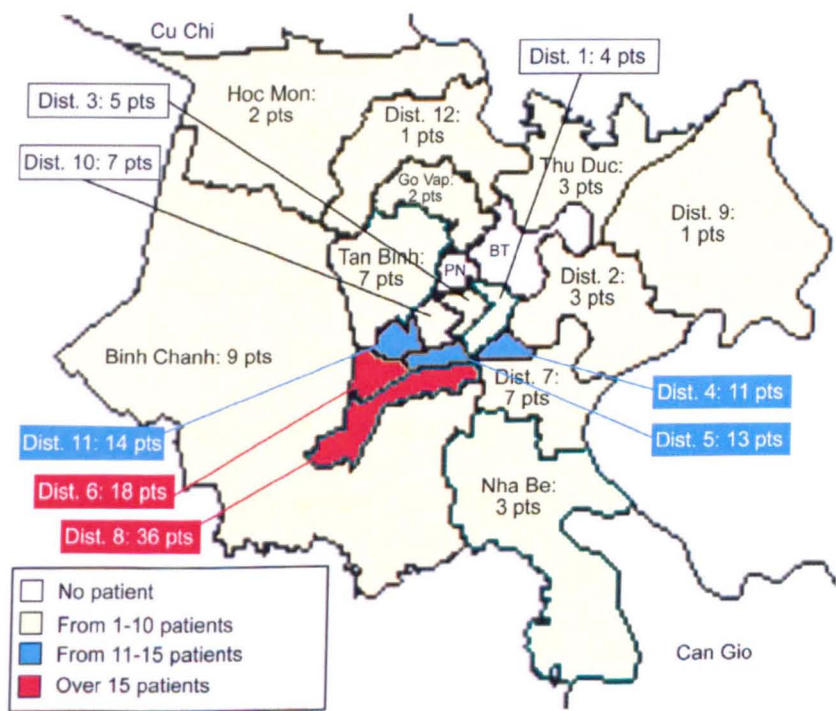
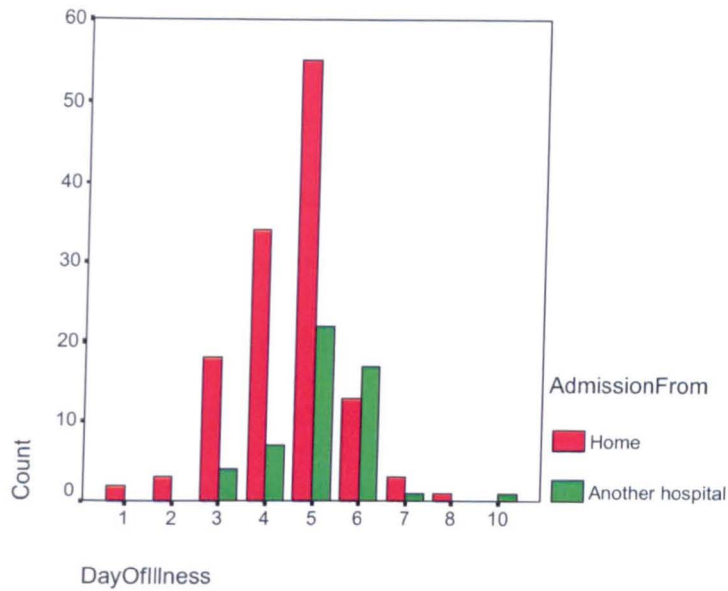


Figure 3.3: Duration of illness (days) prior to admission to HTD.

*Red column: number of patients who admitted to HTD from their home by number of day.*

*Green column: number of patients who admitted to HTD from other hospital by number of day*

#### **3.2.1.2. Symptoms and Signs**

The classic symptoms of dengue, such as fever, headache, vomiting, abdominal pain and bleeding (skin, nose, gum) were present in the history of more than 50% of adults with confirmed dengue (Table 3.2). Fever and bleeding including petechia were the two main symptoms, which brought the patients to the hospital. The spectrum of clinical signs is shown in Table 3.3. External bleeding (petechiae, epistaxis, gingival bleeding) were also present in more than 80% of patients by physical examination. Gastrointestinal bleeding (melena or/and hematemesis) were present in 17% patients with DSS and 21% in DHF without shock. Usually, patients with DHF without shock were treated in the Department of Infection; however, patients with complications, such as gastrointestinal (GI) bleeding were kept under close observation in Intensive Care Unit of our hospital.

In this study, it was necessary to give blood products to nine patients. Two patients, both with severe GI bleeding, prolonged shock, anemia with an Hct drop to below 20%, were transfused by more than 5000 mL of fresh blood. All except one of these patients recovered completely.

There was one patient in the study who died (case fatality =0.5%). This was a man (BC409), 23 yrs, with 4 days of fever, headache, abdominal pain. On day 4, he had melena and admitted to Thong Nhat Hospital in Dong Nai Province for treatment. He was referred to HTD on day 7 with fever, headache, melena, pleural effusion and moderate ascite but with normal blood pressure. Hct was 44%, platelet count  $37900 \times 10^6/\text{dL}$ , leucocyte  $4100 \times 10^3/\text{dL}$ , ALT 50 U/L, AST 40 U/L. The diagnosis of DHF grade II was made. One day later, he suddenly became unconscious with apnea. Cerebral spinal fluid (CSF) shown uncoagulated blood. He was mechanically ventilated for the next 4 days but could not be resuscitated. The final diagnosis was dengue heamorrhagic fever grade II complicated by intracranial bleeding.

Table 3.2: Patients characteristics on history of symptoms and signs before admission

		All groups	DF/DHF I	DHF II	DHF III	DHF IV
<b>Number</b>		N=182	N=4	N=96	N=76	N=6
<b>Gender</b>	Male/Female	98/84	2/2	51/45	42/34	3/3
<b>Fever</b>	No (%)	182 (100%)	4 (100%)	96 (100%)	76 (100%)	6 (100%)
<b>Headache</b>	No (%)	92 (51%)	2 (50%)	47 (49%)	42 (55%)	1 (17%)
<b>Muscle pain</b>	No (%)	22 (12%)	0 (0%)	13 (14%)	9 (12%)	0 (0%)
<b>Vomiting</b>	No (%)	113 (62%)	2 (50%)	57 (59%)	49 (64%)	5 (83%)
<b>Diarrhea</b>	No (%)	26 (14%)	0 (0%)	15 (16%)	10 (13%)	1 (17%)
<b>Abdominal pain</b>	No (%)	92 (51%)	2 (50%)	47 (49%)	40 (53%)	3 (50%)
<b>Bleeding*</b>	No (%)	154 (85%)	0 (0%)	92 (96%)	58 (78%)	4 (67%)

*\*Any kinds of haemorrhage that patient described to doctor, or revealed by examination, including petechia.*

Table 3.3: Clinical features during admission

	All groups	DF/DHF I	DHF II	DHF III	DHF IV
<b>Number</b>	<i>N</i> =182	<i>N</i> =4	<i>N</i> =96	<i>N</i> =76	<i>N</i> =6
<b>Fever on admission</b>	60 (33%)	1 (25%)	32 (33%)	27 (36%)	0 (0%)
<b>Rash</b>	78 (43%)	0 (0%)	44 (46%)	32 (42%)	2 (33%)
<b>Petechia</b>	159 (87%)	0 (0%)	87 (91%)	67 (88%)	5 (83%)
<b>Gum bleeding</b>	51 (28%)	0 (0%)	33 (34%)	17 (22%)	1 (17%)
<b>Epitaxis</b>	21 (12%)	0 (0%)	12 (13%)	9 (12%)	0 (0%)
<b>Bruising</b>	48 (26%)	1 (25%)	24 (25%)	22 (29%)	1 (17%)
<b>GI Bleeding</b>	34 (19%)	0 (0%)	20 (21%)	13 (17%)	1 (17%)
<b>Menorrhagia</b>	17 (9%)	0 (0%)	10 (10%)	7 (9%)	0 (0%)
<b>Hepatomegaly</b>	144 (79%)	2 (50%)	71 (74%)	67 (88%)	4 (67%)
<b>Pleural effusion</b>	73 (40%)	2 (50%)	25 (26%)	42 (55%)	4 (67%)
<b>Pulmonary Edema</b>	2 (1%)	0 (0%)	0 (0%)	2 (3%)	0 (0%)
<b>Hematocrit* %</b>	46	42	45	46	50
<b>Mean (95%C.I)</b>	(45 - 47)	(29 - 55)	(43 - 46)	(45 - 48)	(42 - 58)
<b>Platelet* 10<sup>6</sup>/dL</b>	47000	65333	45739	47139	42600
<b>Mean (95%C.I)</b>	(41625 - 52460)	(20292 - 110373)	(38797 - 52680)	(37580 - 56690)	(25378 - 59821)
<b>Lymphocyte* 10<sup>6</sup>/dL</b>	1546	952	1486	1650	1690
<b>Mean (95%C.I)</b>	(1308 - 1785)	(344 - 1842)	(1260 - 1712)	(1153 - 2149)	(744 - 2638)

\*Result on admission

### 3.2.2 Laboratory results

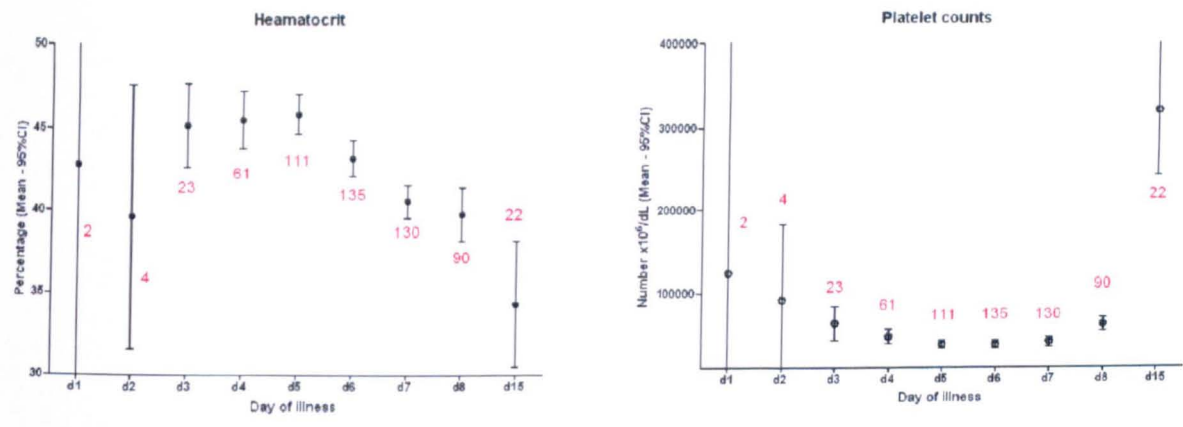
Serum creatinine was in the normal range (mean  $71\mu\text{mol/L}$ , 95% C.I: 67 – 75) in all patients on admission. During hospitalization, blood transaminase levels, alanine transaminase (ALT) and aspartate transaminase (AST) increased greater than upper limits of normal (50 U/L) in 46% and 60% of patients without and with shock syndrome respectively.

The mean haematocrit on admission tended to be greater in DSS patients than in DHF I & II (Table 3.3). The trend of Hct was increased from day 2 of the illness to day 3, 4 and up to the peak at day 5 then dropped in recovery phase (figure 3.4). In term of criteria of haematocrit threshold of plasma leakage by WHO, there were only 43% of patients with DHF grade I and II, 63% of patients with grade III and IV meet that criteria in our study (who had haematocrit on admission greater than 45%) .

Means of platelet count on admission tended to be lower in DSS groups than in DHF I & II (Table 3.3). On admission, thrombocytopenia (platelet count  $< 100,000/\text{mm}^3$ ) was present in 91% of patients without shock and in 89% patients with shock syndrome. Therefore, there were approximately 10% of patients who did not meet WHO criteria for thrombocytopenia on admission, even in the DSS group. Platelet counts generally declined from day 1 of the illness to day 5, then recovered slowly in almost of the patients, platelet count bounced back to the normal range within 2 weeks of illness onset (figure 3.4). During hospitalization, marked thrombocytopenia (platelet count  $< 50,000/\text{mm}^3$ ) occurred in 72% of patients without shock and in 71% patients with shock syndrome.



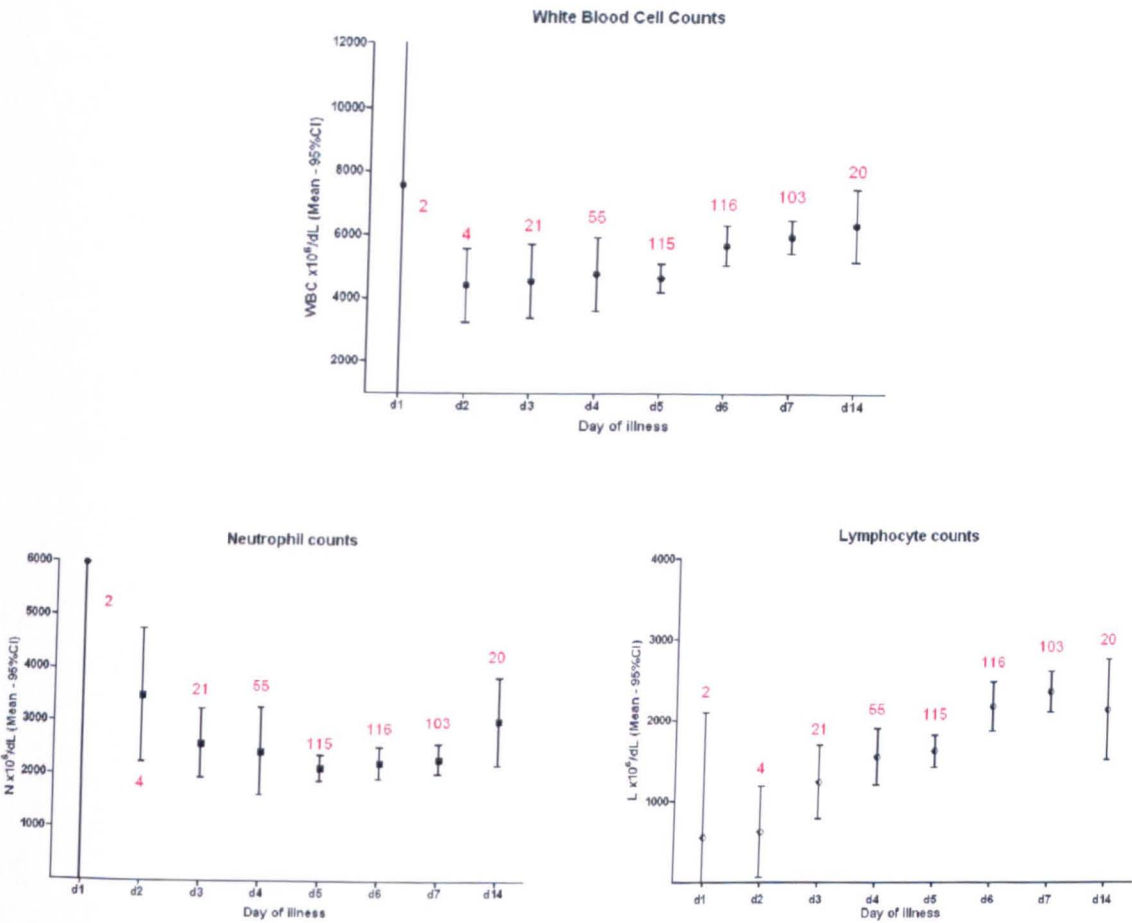
Figure 3.4: Distribution of Heamatocrit and Platelet counts by day of illness of all patients.



Plotted with total number of cases, mean with 95% confidential interval.

Leucocytes, neutrophils had trend of the decline from day 1 of the illness over first week then recovered slowly in recovery phase. Leucocyte counts were still lesser than  $8000 / \text{mm}^3$  at day 14 after the onset. Lymphocyte counts that were low on admission with mean was  $1546 / \text{mm}^3$  (95%CI= 1308 – 1785), tented to be increased over acute phase (figure 3.5).

Figure 3.5: Evolution of Leucocyte, neutrophil and lymphocyte counts by day of illness



Plotted with total number of cases, mean with 95% confidential interval

3.2.3 Cytokine investigation

A subset of patients that included 58 DHF grade I and II patients and 18 DSS patients had plasma samples for cytokine measurements. An acute blood sample was collected into EDTA from patients who gave consent as soon as possible after enrolment into the study. Blood was separated promptly and plasma was stored at -70°C. A second blood sample, taken upon hospital discharge, served as the

convalescent phase sample. Concentrations of TNF $\alpha$ , soluble TNF receptors, IL-6 and IL-10 from acute and convalescent samples were measured and the results are shown in Table 3.4.

Mean TNF $\alpha$  levels on admission were 38 pg/mL (95% CI, 32 to 44) in DHF grade I & II group and 54 pg/mL (95% CI, 17 to 91) in DSS group. In comparison between acute and convalescent paired samples from each individual, TNF $\alpha$  plasma levels declined to significantly lower levels in convalescent samples irrespective of disease severity ( $p < 0.05$ ). The concentration of soluble TNF receptor p55 and p75, IL6 and IL10 showed a positive trend (as shown in Figure 3.6) between mild diseases (DHF I&II) to more severe diseases (DSS), although there was not a statistically significant difference between two groups. Except for soluble TNF receptor p55 in the DSS group, there were elevated concentrations of all studied cytokines in the acute phase (on admission) in compared with its concentrations from convalescent samples (at discharge of the hospital) in each individual (Table 3.4).

Of the 58 patients with DHF I&II, 29 (50%) had detectable levels of IL-6 and IL-10 in serum. There were significantly higher levels of IL-6 (mean, 37 pg/ml; 95% C.I, 24 to 50 pg/ml) and IL-10 (mean, 38 pg/ml; 95% C.I, 26 to 50 pg/ml) in acute samples than in convalescent phase (mean, 13 pg/ml, and 95%C.I, 10 to 18 pg/ml) and (mean, 18 pg/ml, and 95%C.I, 10 to 25 pg/ml), respectively.

Of the 18 patients with DSS, 11 (61%) and 9 (50%) had detectable levels of IL-6 and IL-10 in serum, respectively. Acute levels of IL-6 (mean, 53 pg/ml; 95% C.I,

15 to 92 pg/ml) and IL-10 (mean, 34 pg/ml; 95% C.I, 13 to 54 pg/ml) were significantly higher than in convalescent samples.

Table 3.4: Levels of Cytokines & Chemokines in DHF I&II group versus DSS groups

	DHF I&II	(N=58)	P*	DSS	(N=18)	P value*
	Acute	Convalescent		Acute	Convalescent	
<b>TNF (pg/mL)</b>	38	25	<0.01	54	23	<0.05
<i>Mean (95%C.I)</i>	(32 – 44)	(19 – 30)		(17 - 91)	(15 – 32)	
<b>sTNFR55 (pg/mL)</b>	1882	1466	<0.01	2351	1967	NS
<i>Mean (95%C.I)</i>	(1723 – 2041)	(1325 – 1606)		(1644 - 3057)	(1509 - 2425)	
<b>sTNFR75 (ng/mL)</b>	12.8	7.6	<0.01	21.1	11.5	<0.05
<i>Mean (95%C.I)</i>	(10.9 – 14.6)	(5.9 – 9.2)		(12.9 – 29.2)	(7.4 – 15.5)	
<b>IL 6 (pg/mL)</b>	37	13	<0.01**	53	17	<0.01***
<i>Mean (95%C.I)</i>	(24 – 50)	(10 – 18)		(15 – 92)	(10 – 26)	
<b>IL 10 (pg/mL)</b>	38	18	<0.01**	34	10	<0.05*+*
<i>Mean (95%C.I)</i>	(26 – 50)	(10 – 25)		(13 – 54)	(0 – 17)	

P value\*: Wilcoxon signed rank test.

\*\*.: comparison 29-paired values that were above detection limit (10 pg/mL).

\*\*\*.: comparison 11-paired values that were above detection limit (10 pg/mL).

\*+\*.: comparison 9 paired values that were above detection limit (10 pg/mL).

Figure 3.6: Levels of Cytokines

Figure 3.6.a: TNFa levels.

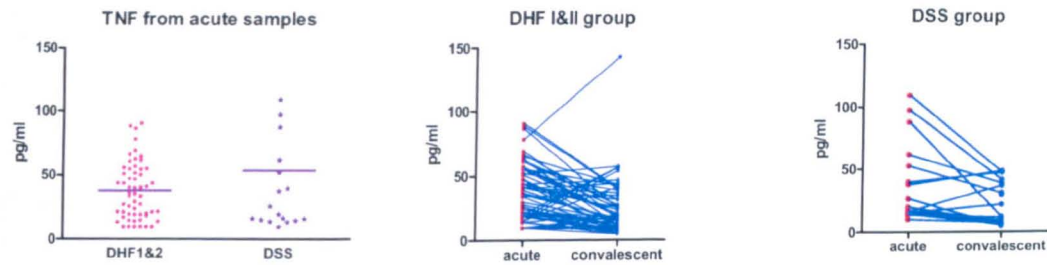


Figure 3.6.b: Soluble TNF receptor 55 levels.

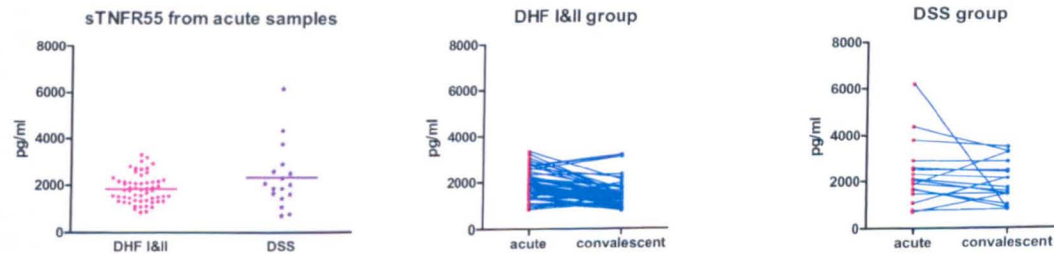


Figure 3.6.c: Soluble TNF receptor 75 levels.

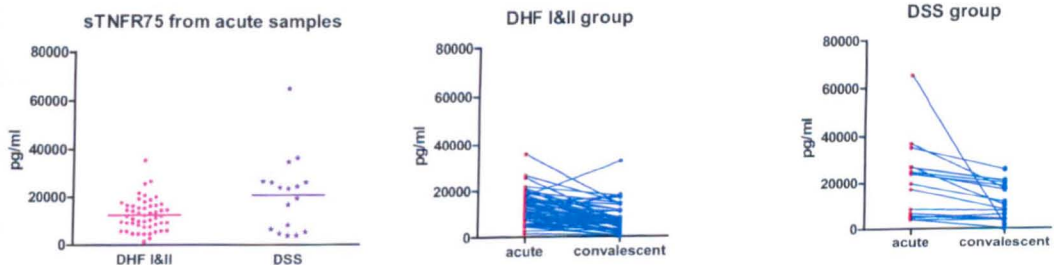
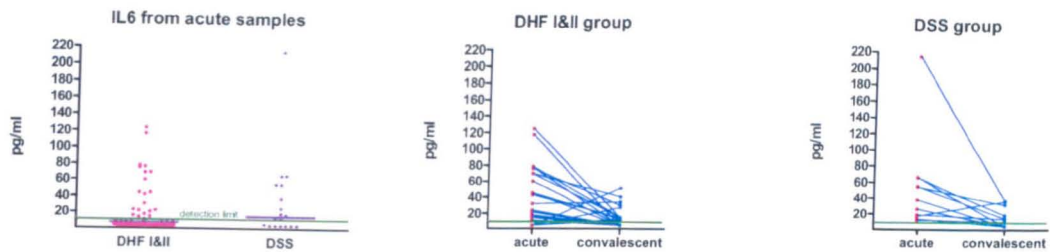
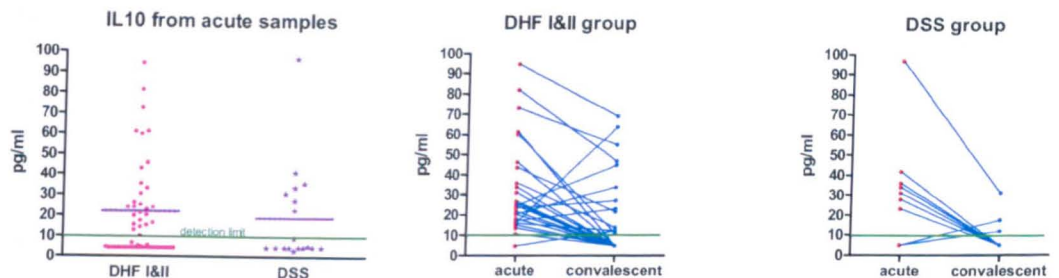


Figure 3.6.d: *Interleukin 6 levels.*Figure 3.6.e: *Interleukin 10 levels.*

Left charts: cytokine levels from acute samples, plotted by groups of severity – DHF I&II versus DSS.

Middle and Right charts: cytokine levels in paired samples - acute versus convalescent samples, plotted by DHF I&II and DSS groups, respectively.

### 3.2.4 Management and Outcome: data from all death in the Hospital for Tropical Diseases (1997 – 2006)

There was one patient with DHF grade II complicated by intracranial bleeding died in this descriptive study. As this is not intervention trial, it is difficult to interpret the management measures.

However, we had kindly permission of having the data of 28 adults with DHF/DSS who died in HTD between 1997 to 2006 (in those, 61% had serological evidence of dengue infection). In this period, there were 10861 adults who admitted to HTD

with DHF/DSS, therefore the in-hospital case fatality was 0.26%, was higher than in children in the same period of 0.09% (source: HTD).

There were 12 males and 16 females (ratio was 0.75:1). The mean age was 25 years (SD=8), the range of age from 16 to 49 years. Amongst the 28 patients, 9 patients (32%) lived in Ho Chi Minh City and 19 (68%) in other provinces of Southern Viet Nam.

Only 4 patients admitted directly to HTD on the 4.5<sup>th</sup> day after onset (SD: 0.6; range: 4 - 5). The majority of 24 patients were referred to HTD from another hospital. The average stay in other district / provincial hospitals where they received treatment was 34 hours (SD: 36 hrs; range: 2 – 144 hrs). Therefore, those 86% of this study population admitted to HTD on the 6<sup>th</sup> day after onset (SD: 1.6; range: 4 - 10). There were 23 patients had sustained shock before admission at the HTD, these patients were treated at previous hospital (district / provincial levels) during 35 hours (range: 2 – 44y hrs). On admission at the HTD, there were 13 patients who had fluid overloading, in those 6 patients with DSS and 7 without shock syndrome.

Patients were treated in HTD for a mean duration of 34 hours (range: 1 - 96). 13 patients were necessary of blood transfusion, minimum amount was 250 mL and maximum amount was 3750 mL of fresh whole blood.

Complication of metabolic acidosis were in 5 patients with prolong DSS, in 5 patients with suspected intracranial bleeding, in 16 with severe bleeding such as gastrointestinal bleeding, disseminated intra-vascular coagulopathy. There were 11

patients had the combination of shock and severe bleeding on admission. 8 patients died very quickly after admission (from 1 to 5 hours).

### **3.3 Discussion**

#### **3.3.1 Clinical features**

DHF has been primarily a disease of children ((PAHO) 1994) ((WHO) 1997). Reports from 1975 to 1978 from Southeast Asia where DHF is endemic revealed only eight of 629 patients in Indonesia and 18 of 694 in Thailand to be older than 15 years (Nimmannitya 1987) (Sumarmo, Wulur et al. 1983).

In the outbreaks in the 1980s in Cuba and Venezuela, the disease occurred in all age groups, although about two-thirds of the fatalities were among children (Kouri, Guzman et al. 1989) (Guzman, Alvarez et al. 1999). Similar observations have been made in Brazil and Puerto Rico in the 1990s (Zagne, Alves et al. 1994) (Rigau-Perez 1997).

Recently, the age distribution of DHF cases in Asia has changed progressively. The number of DHF adult cases has been increased during the 1980s and the 1990s in Southeast Asia countries such as Philippines, Malaysia (Capeding, Paladin et al. 1997) (Ibrahim and Cheong 1995).

The ratio of adult:children DHF cases has increased since the 1990s in the Hospital for Tropical Diseases (HTD), Ho Chi Minh city, Viet Nam (Figure 1.3). Data from HTD showed that between 1997 to 2006, the in-hospital case fatality rate of adults was higher than in children (0.26% vs. 0.09%). As a referral hospital for the south



of Viet Nam, the population being studied in this project may not be representative of the case burden in the whole of Southern Viet Nam.

In this present study, a high proportion of patients with DHF had abdominal symptoms: vomiting (62%), diarrhoea (14%), and abdominal pain (51%). This features is more similar to data from Cuba in 1997 that showed 100% adult DHF patients had vomiting and 51% had abdominal pain (Guzman and Kouri 2002). Sometimes, abdominal pain simulated an acute surgical abdomen. Patients who had severe abdominal pain, vomiting associated with little amount of ascite were misdiagnosed as acute surgical abdomen and were initially treated in a surgical hospital until they later developed skin bleeding and severe hypotension. Those misdiagnosed cases with high mortality due to severe bleeding had been reported in some hospitals in Viet Nam (data from HTD). Dengue infections presenting with predominantly gastro-intestinal symptoms also have been reported recently in outbreak 2002 in Singapore (Seet, Ooi et al. 2005) and therefore, highlight the need for medical personnel to consider the possibility of dengue when assessing patients from areas where it is common, especially during a major dengue epidemic.

Bleeding is other important sign of DHF. In our current study, bleeding site included petechia (87%), gums (28%), bruising (26%), gastro-intestinal tract (19%), nose (12%) and vaginal (9%). In other studies of hospitalized patients in Malaysia, haemorrhagic manifestations were observed in 62% to 68.8% (Wallace, Lim et al. 1980) (Shekhar and Huat 1992). Recent studies from Taiwan revealed

that the incidence of gastrointestinal bleeding varied from 11.1% to 58.5% (Wang, Tseng et al. 1990) (Tsai, Kuo et al. 1991).

The fact that internal bleeding with a huge volume occurred acutely but without obvious external blood loss might causes recurrent or persistent shock, whereas the haematocrit may not decline due to haemoconcentration, adds to the difficulty of making a diagnosis and giving the appropriate treatment. It is thus recommended that blood transfusion should be given promptly if there is suspected internal heamorrhage even though the haematocrit may remain apparently stable.

One patient in the present study died with the dramatic clinical picture of intracranial haemorrhage. Encephalopathy in DHF could result from cerebral anoxia, oedema, intracranial haemorrhage, and vessel occlusion. In a previous study in our hospital of 378 Vietnamese patients with suspected central nervous system infections, 4.2% were infected with dengue viruses. The most frequent neurological manifestations of this group were reduced consciousness and convulsions. Nine patients had encephalitis. No patient died, but six had neurological sequelae at discharge (Solomon, Dung et al. 2000). With the development of more sensitive molecular diagnostic methods, the chance of dengue detection from cerebrospinal fluid or brain tissue has increased; however, the question of contamination with blood has been raised or passage of the dengue virus through a leaky blood brain barrier. It is possible that haemorrhage or leakage through the blood-brain barrier enables antibody and virus present in the blood to move into the cerebrospinal fluid, or dengue virus could cross the blood-

brain barrier and infect the cerebrospinal fluid. Therefore, more pathological studies are needed.

### **3.3.2 Dengue classification and case definitions**

Recommendations for the classification and management of DHF were developed following key findings in Bangkok in the 1960s. In particular, a study in 1964 of 123 Thai children admitted to hospital with dengue, researchers identified that clinically important loss of fluid from the vascular compartment was indicated by a 20% increase in haematocrit (Cohen and Halstead 1966). The recommendations evolved into the WHO guidelines of 1974, updated in 1986, 1994, and 1997 ((WHO) 1997). Undoubtedly, widespread adoption of the WHO system, together with implementation of the accompanying management guidelines, contributed to the striking initial fall in case-fatality rates. However, after the recent global expansion in dengue disease, several groups in Asia and Latin America have reported difficulties in using the system (Bandyopadhyay, Lum et al. 2006).

In this present study, an elevated haematocrit above 20% of baseline (i.e. haematocrit  $\geq 45\%$ ) as indicator of hemoconcentration was only present in 43% and 63% of patients on admission without and with DSS, respectively. Moreover, there were also 10% of patients who did not meet WHO criteria for thrombocytopenia on admission, even in the DSS group.

In terms of management, WHO clinical criteria are inadequate to classify adult Vietnamese patients, particularly those with GI bleeding. As a consequence, blood transfusion might be delayed.

In a study of virologically confirmed dengue deaths in Jakarta, Indonesia (Sumarmo, Wulur et al. 1983), nine (30%) patients had gastrointestinal bleeding before the onset of shock with no evidence of haemoconcentration during the hospital stay, so they did not meet the WHO criteria for DHF. Reports from the Philippines (Hayes, Manaloto et al. 1988) and Kuala Lumpur (Ibrahim and Cheong 1995) also showed significant haemoconcentration in only 39.5% and 22% of the patients respectively.

In a recent study in Viet Nam (Phuong, Nhan et al. 2004) of the 712 children with confirmed dengue infection, bleeding and thrombocytopenia were almost as common in the 312 children classified as having dengue fever and in the 319 classified as having DHF. In addition, of the 310 children with shock and laboratory-confirmed dengue, 57 (18%) did not meet all four WHO criteria for DHF and were thus classified as having DF by default. Among those, 22 failed because they had a negative tourniquet test result and no bleeding manifestations throughout the hospital stay, 15 because the platelet count was never less than  $100,000/\text{mm}^3$  and the remaining 20 because they did not reach the predefined hematocrit cut-off value of  $\geq 45\%$ .

Current WHO classification system and case definitions distinguishes rigorously between dengue fever, DHF, and DSS, but there is much overlap between the three. Possibly, as previously suggested (Gubler 1998), dengue disease exists as a continuous range rather than as distinct clinical entities. In addition, this system excludes severe dengue disease associated with “unusual manifestations”. New

categories as “dengue fever with unusual hemorrhagic manifestations” or “dengue with signs associated with shock” have appeared. Therefore, a large multicentre descriptive study is needed to obtain the evidence to establish a robust dengue classification scheme for use by clinicians, epidemiologists, public-health authorities, vaccine specialists, and scientists involved in dengue pathogenesis research. Dengue case definitions derived in this way might prove more useful for presumptive diagnosis, management, and final diagnosis, than the existing scheme (Deen, Harris et al. 2006).

Recently, a revised system of dengue classification was proposed at an October 2008 meeting of experts at WHO headquarters, Geneva, Switzerland. The revisions are based upon a review of clinical data from multicentre studies known as “DENCO” (DENGue COntrol) in seven countries across Southeast Asia (included Hospital for Tropical Diseases, Ho Chi Minh city Viet Nam), Latin America and Western Pacific, which was supported by TDR/WHO with funding by the European Union and the Wellcome Trust. This new simplified dengue classification system that groups dengue cases into just two major categories of severity – dengue (with or without warning signals) and severe dengue, will now be tested and validated in studies in dengue endemic regions over the coming years (TDRNews No81 November 2008).

### **3.3.3 Plasma leakage**

The cardinal feature in DHF is a transient increase in vascular permeability that results in the leakage of fluid from the plasma to the interstitium (Halstead 1989).

The circulating blood volume is reduced, hemoconcentration occurs, and, in severe cases, hypovolemic shock develops. After a few days, the increase in permeability resolves spontaneously, the leaked fluid is reabsorbed, and the patient recovers quickly.

Microvascular permeability can be assessed noninvasively in humans by use of mercury-in-silastic strain gauge plethysmography (MSG). The MSG assessments were made at the midcalf of thigh and analysis of a continuous recording of limb circumference, obtained during a specific venous congestion pressure protocol, enables the quantitation of the changes in peripheral microvascular parameters, including permeability and blood flow (Gamble, Gartside et al. 1993).

In study of 89 healthy Vietnamese subjects aged 5 to 77 years, the mean coefficient of microvascular permeability ( $K(f)$ ) was highest in the youngest children then 3- to 4-fold lower towards the end of the second decade (Gamble, Bethell et al. 2000). Moreover, other study of Vietnamese children with DSS ( $n = 19$ ), DHF without shock ( $n = 16$ ), and healthy control children ( $n = 15$ ) showed that the mean of  $K(f)$  for the patients at admission and after fluid resuscitation was approximately 50% higher than that for the control patients ( $P=0.02$ ). There was no significant difference in  $K(f)$  between the 2 groups of patients - DHF without shock and DSS groups; this suggests the same underlying pathophysiology (Bethell, Gamble et al. 2001). Perhaps the fluctuations in  $K(f)$  in patients with DSS are larger than those in patients with DHF, which leads to short-lived peaks of markedly increased microvascular permeability and consequent hemodynamic

shock. Young mammals are known to have a larger microvascular surface area per unit volume of skeletal muscle than adults. During development, the proportion of developing vessels is greater. In addition, the novel microvessels are known to be more permeable to water and plasma proteins than when mature. These factors may explain for the age-related trend that children more readily develop hypovolaemic shock than adults in dengue haemorrhagic fever and other conditions characterized by increased microvascular permeability.

### **3.3.4 Cytokine profiles and plasma leakage**

Our present study has shown an association between elevated cytokine levels and the acute phase of dengue. Overall, plasma concentrations of TNF $\alpha$ , sTNFR75 IL6, IL10 in both groups (DHF I&II and DSS) and sTNFR55 in DHF I&II group were significantly higher at acute presentation compared with convalescent values but not sTNFR55 levels of DSS group.

However, the study has failed to demonstrate the significantly statistical difference of cytokine levels between DHF without shock and DSS groups. It is likely affected by the capillary leak. Plasma leakage that possibly resulted from an alteration in the function of the endothelial glycocalyx during dengue infection, contributes significantly to the reduction in plasma concentrations of different proteins (Michel and Curry 1999). The leakiness of each molecule is determined by complex factors; based on molecular weight alone, it has been showed that the smaller proteins such as human albumin (molecular weight [MW], 66.5 kDa) and transferrin (MW, 79 kDa) were more affected than IgG (MW, 150 kDa) (Wills,

Oragui et al. 2004). Therefore, human TNF $\alpha$  (MW, 51 kDa), sTNFR-55 (MW, 25 kDa), sTNFR-75 (MW, 35 kDa), IL-6 (MW, 26 kDa) and IL-10 (MW, 18.6 kDa) are likely to be affected by this capillary leak. The more severe disease, the more cytokine leakage. In consequence, clinical study that aims to compare the concentration of plasma proteins in different severity groups of dengue infection would be adjusted by correcting and providing an approximation of the level that might have been achieved in the absence of vascular leakage. The extend of this leakage can be gauged by the effect on albumin and heamatocrit which are apparently indirect markers of vascular permeability. Unfortunately, the leakiness of each molecules is also affected individually by other factors such as its electronic charge, magnitude of alteration of electronic charge and damage extent on the capillary wall (Wills, Oragui et al. 2004). Therefore, interpretation of statistical association between plasma protein (e.g. cytokine, NS1 protein, etc) and severity of disease would be performed with carefulness in those group of diseases in which hypovolemic shock secondary to vascular leak commonly occurs such as DHF.

Data from Malaysia and Tahiti have suggested that the level of TNF $\alpha$  production in DHF may be related to clinical outcome (Yadav, Kamath et al. 1991) (Hober, Poli et al. 1993).

The association of TNF $\alpha$  with disease severity has been confirmed independently in mouse model of dengue infection (Atrasheuskaya, Petzelbauer et al. 2003). In



this experiment, increased TNF $\alpha$  levels were detected in sick animals and antibodies to TNF $\alpha$  attenuated the disease phenotype.

The combination of evidence of increased levels of TNF  $\alpha$ , soluble tumor necrosis factor receptor 55 and 75 in acute phase of DHF adult patients in our study together with previously similar finding elsewhere (Hober, Delannoy et al. 1996) (Bethell, Flobbe et al. 1998) (Braga, Moura et al. 2001) and evidence in animal studies, strongly suggested the implication of TNF $\alpha$  in the pathogenesis of dengue infection.

Both type 1 (p55) and type 2 (p75) receptors for human TNF $\alpha$  (TNFR) may exist on the cell membrane as a signal-transducing unit or in a soluble form in the extracellular fluid (sTNFR). Soluble receptors compete with membrane-bound receptors for TNF binding. High amounts of soluble TNF receptors function as specific inhibitors of TNF activity on target tissues. However, it should be noted that TNF receptors may, under certain circumstances, function as TNF agonists rather than TNF antagonists (Mohler 1993). The sTNFR-75 may bind to TNF $\alpha$  in the circulation and prolong its circulating half-life. Because TNF $\alpha$  may readily dissociate from the type 2 receptor, the end result may be prolongation of TNF activity in the systemic circulation with potentially detrimental effects (Evans 1994).

Elevated sTNFR levels could theoretically be due to increased production or to reduced clearance. The latter has been noted in renal failure due to septic shock (Froon, Bemelmans et al. 1994), but from our study, renal impairment is

uncommon in nonfatal DSS. We consider a more likely explanation to the increased shedding of sTNFR in our study due to proinflammatory stimulation. It may be relevant that the strong clinical association was observed for sTNFR-75, since this form of sTNFR appears to be preferentially over expressed on microvascular endothelium in persons with acute respiratory distress syndrome (Grau, Mili et al. 1996), and had consistent positive relationship with disease severity (Bethell, Flobbe et al. 1998). Possibly, sTNFR levels in DHF reflect inflammatory events at the endothelial level and might therefore constitute a specific marker of incipient microvascular pathology.

A number of cytokines besides TNF $\alpha$  have been implicated in DHF/DSS, most commonly INF $\gamma$ , IL-6, and IL-10. Interleukin (IL)-10 is a cytokine that inhibits the production of a wide range of inflammatory and proinflammatory cytokines. For example, human IL-10 suppresses the expression of IL-2, IL-3, interferon- $\gamma$ , and granulocyte-macrophage colony-stimulating factor in T helper type 1 cells (Fiorentino, Bond et al. 1989; Fiorentino, Zlotnik et al. 1991); IL-4 and IL-5 in T helper type 2 cells (Moore, O'Garra et al. 1993); IL-8 and macrophage inflammatory protein-1 in neutrophils (Kasama, Strieter et al. 1994); IL-1, IL-8, and TNF $\alpha$  in macrophages/monocytes (Howard and O'Garra 1992) (Bogdan, Vodovotz et al. 1991). Hence, IL-10 is a potent anti-inflammatory cytokine that has an important role in balancing inflammatory responses (Wang, Wu et al. 1994).

In this study, pro-inflammatory plasma cytokine IL-6 and anti-inflammatory plasma cytokine IL-10 levels were significantly higher at acute presentation compared with convalescent phase in both groups (DHF I&II and DSS). IL-6 and IL-10 has been found in some previous studies and significantly elevated in DHF versus DF patients (Hober, Poli et al. 1993) (Green, Vaughn et al. 1999). There is association of increased levels of IL-10 in dengue patients with a sequential infection (Perez, Garcia et al. 2004). IL-6 and IL-10 may play a role in the pathogenesis of DHF/DSS in infants (Nguyen, Lei et al. 2004).

The pro-inflammatory response is regulated by the anti-inflammatory components of the immune system (Gomez-Jimenez, Martin et al. 1995). A complex network of cytokines is generated in response to a systemic immune challenge. It is the net effect of interactions between these proinflammatory and anti-inflammatory molecules over time that determines the nature of the immune response in individual patients. An imbalance between these two aspects of the inflammatory response may be deleterious. Elevated plasma IL-10 levels and raised IL-10 to TNF $\alpha$  ratios have been associated with poor outcome and increased mortality in sepsis (Lehmann, Halstensen et al. 1995), (van Dissel, van Langevelde et al. 1998), it is more likely to have fatal infections in meningococemia (Westendorp 1997). Similarly, patients with high ratios of TNF to soluble TNF receptors are at increased risk of having lethal meningococcal infections (Girardin 1992). Taniguchi et al. found that an increase in the ratio of IL-6 to IL-10 correlated with a poor outcome in patients with systemic inflammatory response syndrome (SIRS) (Taniguchi, Koido et al. 1999). It therefore seems that both over-robust and

inadequate anti-inflammatory responses can be associated with a poor prognosis, although insufficient data are available to define an appropriate response in any individual context.

The unresolved question is why capillary leak is such a prominent feature of DHF when a variety of other infectious diseases show large elevations in TNF and other proinflammatory cytokines without profound alterations in capillary permeability. The pathophysiological mechanisms underlying the capillary leak in dengue infection are poorly understood. Many factors including total virus burden, viral virulence, host immune response (Bethell, Flobbe et al. 1998) (Leitmeyer, Vaughn et al. 1999) (Vaughn, Green et al. 2000), and genetic predisposition (Stephens, Klaythong et al. 2002) have been implicated in the pathogenesis of DHF, but the means by which any of these factors might influence endothelial function remain controversial.

Endothelial cells can be infected by dengue virus in vitro (Bunyaratvej, Butthep et al. 1997) (Avirutnan, Malasit et al. 1998) (Diamond, Edgil et al. 2000) (Huang, Lei et al. 2000), but the endothelium of dengue haemorrhagic fever patients shows little evidence of infection or endothelial cell damage due to infection (Andrews, Theofilopoulos et al. 1978) (Sahaphong, Riengrojpitak et al. 1980) (Killen and O'Sullivan 1993) (Kurane 1997). This suggests the endothelium is not a site for direct viral replication or cytolysis in vivo; however, alterations in endothelial cell permeability induced after infection of other cell types may contribute to vascular leakage.

There are many known modulators of endothelial cell permeability, included cytokines, thrombin, cytotoxic factors, sVCAM-1, that regulate the endothelium to maintain an active barrier between blood and tissues (Royall JA 1989) (Rabiet M-J 1996). Cytotoxic factor has been described that is produced from dengue virus-infected peripheral blood mononuclear cells (PBMC) is present in dengue patient sera (Shaio, Cheng et al. 1995; Agarwal, Chaturvedi et al. 1998), and can induce leakage in the peritoneal cavity of mice. Peak leakage occurred 30 min after inoculation of cytotoxic factor and the vascular integrity was restored by 2 hours (Khanna, Chaturvedi et al. 1990). TNF- $\alpha$  released from dengue virus-infected monocytes in supernatants can induce TNF- $\alpha$  dependent activation of endothelial cells, as measured by upregulation of endothelial cell expression of VCAM-1 and ICAM-1 (Anderson, Wang et al. 1997). Plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration, markers of endothelial damage, were significantly higher in severe disease of dengue haemorrhagic fever (Murgue, Cassar et al. 2001). Similarly, studies with Marburg virus, which causes a more severe haemorrhagic fever than dengue virus, have shown that supernatants from infected monocyte-derived macrophages can rapidly induce TNF- $\alpha$  dependent changes in endothelial cell permeability (Feldman H 1996). Recently, Lin et al. showed that in vitro, antibodies against dengue virus nonstructural protein 1 (NS1) act as autoantibodies that cross-react with noninfected endothelial cells and trigger the intracellular signaling leading to the production of nitric oxide and to apoptosis (Lin, Lei et al. 2002). Activation and damage of human microvascular endothelial

cells could be induced in vitro by inflammatory mediators present in sera from patients with acute dengue infection (Cardier, Marino et al. 2005).

The difference in pathogenesis of prominent feature of cytokine - associated capillary leakage in dengue infection and non-profound alterations in capillary permeability in other systemic infections, that all show large elevations in cytokines, could be possibly explained from the result of Carr's experiment. In vitro, Carr et al. showed that supernatants from dengue virus type 2-infected monocyte-derived macrophages increased permeability in human umbilical vein endothelial cell monolayers without inducing endothelial cell infection. Interestingly, in their experiment, induction of endothelial cell permeability by supernatants from dengue virus infected monocyte-derived macrophages did not coincide with the timing at the peak of TNF-alpha release from the latter cells. Permeability changes in endothelial cells were observed with supernatants from dengue virus infected monocyte-derived macrophages at 96 hr post infection, whereas the peaks of TNF-alpha release were biphasic, initial response at 4–24 hr after mock infection - that was likely a result of cell manipulation and second peak at 36–48 hr post infection - that represents a viral specific response. Additionally, pre-exposure of endothelial cells to supernatants harvested at the time of peak release of TNF $\alpha$ , enhanced the subsequent endothelial cell permeability induction (Carr, Hocking et al. 2003). Clinically, this phenomenon may be compared to the pathophysiology of dengue haemorrhagic fever where the peak of plasma leakage occurs late in the course of disease, during or after the decline in fever.

Therefore, we speculated that the mechanisms of endothelial cell permeability in DHF may involve to endothelium activation induced by TNF-alpha release from dengue virus-infected monocytes then followed by action of other factor(s), it is possibly by other types of cytokine or more likely, by another pulse of proinflammatory mediators released into the peripheral circulation in later phase of immune responses. Pulse(s) of proinflammatory mediators released into the peripheral circulation from the process of immune responses of dengue infection would result in sudden, rapid, massive, but self-limiting, hypovolemic shock. Repeated episodes of shock could be explained by further pulses of circulating mediators. Further studies on endothelial cell permeability in dengue infection and other diseases that had elevated cytokines are needed.

In term of management and outcome of dengue infection, plasma leakage would allow plasma water to flood out of the intravascular compartment, that would result in hemoconcentration, ascites, and pleural effusions, and when the compensation mechanism is overridden, that would lead to hypovolemic shock. Failure to treat shock at this early stage leads to the development of severe shock disease, whereby the patient becomes into uncompensated shock syndrome with complications of metabolic acidosis, disseminated intravascular coagulopathy, edematous and had poor outcome. Unfortunately, the regimen and volume of fluid replacement for adult with DHF/DSS remain empirical and are not described in the World Health Organization Management Guidelines. In most of cases, the resuscitation was based on the regimen of hypovolemic shock (such as severe acute diarrhea) or septic shock or even based on DHF/DSS children's WHO

guidelines. This might explained such number of patients with fluid overloading and complication of pulmonary acute edema in the past. Based on ideal body weight and WHO guidelines (for children), clinicians from HTD has established the empirically volume replacement guideline for adult with different grades of DHF and it has been implemented into Viet Nam National Guideline for Dengue Heamorrhagic Fever. The preliminary effect on overall reduction of severe mortality and case fatality after implication of new guideline to all hospitals in Southern Viet Nam was presented in the annual academic conference of HTD in 2005. Further large-scale studies of the volume replacement and resuscitation are needed.



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**CHAPTER 4**

**Cellular immune responses**

**in dengue patients**

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#### **4.1 Introduction**

Epidemiological studies suggest the frequency of DHF is substantially higher in individuals experiencing a secondary infection with a heterologous dengue serotype (Sangkawibha, Rojanasuphot et al. 1984; Burke, Nisalak et al. 1988). While children carry most of the symptomatic disease burden in endemic countries, young adults are also significantly affected. Several explanations have been proposed to explain the epidemiological association between DHF and secondary infection with a heterologous dengue virus. Viral, host genetic and nutritional factors have been associated with susceptibility to DHF (Thisyakorn and Nimmannitya 1993) (Rico-Hesse, Harrison et al. 1997) (Leitmeyer, Vaughn et al. 1999) (Gamble, Bethell et al. 2000) (Loke, Bethell et al. 2001) (Loke, Bethell et al. 2002) (Stephens, Klaythong et al. 2002).

Cytotoxic T-lymphocytes (CTLs) play a major part in the cellular immune response to viruses and other intracellular infections. A role for T lymphocytes in DHF pathogenesis has been discussed (Kurane 1997). CD8<sup>+</sup> T cells may contribute both to protection against and to development of DHF. A protective role for CD8 T cells is plausible, because these cells may limit viral infection by cytolysis of infected cells and secretion of antiviral cytokines such as IFN $\gamma$ .

However, the mechanism by which T cells contribute to DHF pathogenesis is less clear, although new insights have been acquired in the last few years. Serotype cross-reactivity is likely to be important and has been possibly implicated in the immunopathogenesis of dengue infection. Primary dengue virus infections induce

serotype-specific and serotype-cross-reactive, CD4+ and CD8+ memory cytotoxic T lymphocytes (CTL). In secondary infections with a virus of a different serotype from that which caused primary infections, the presence of cross-reactive non-neutralizing antibodies results in an increased number of infected monocytes by dengue virus--antibody complexes. This in turn results in marked activation of serotype cross-reactive CD4+ and CD8+ memory CTL. It is hypothesized that the rapid release of cytokines and chemical mediators caused by T cell activation and by CTL-mediated lysis of dengue virus-infected monocytes triggers the plasma leakage and hemorrhage that occurs in DHF (Kurane, Rothman et al. 1994).

CD4+ and CD8+ T cell responses to dengue virus in both mice (Rothman, Kurane et al. 1996) (Spaulding, Kurane et al. 1999) and humans (Mathew, Kurane et al. 1998), (Zivny, Kurane et al. 1995) with cross-reactivity demonstrated to both completely and incompletely conserved epitopes. It had been shown that activated dengue serotype cross-reactive memory CD4+ T lymphocytes secrete cytokines including  $\text{TNF}\alpha$  and  $\text{IFN}\gamma$ , which might contribute to the enhanced pathology manifested as DHF (Gagnon, Ennis et al. 1999).

Inflammatory cytokines secreted by CD8+ T cells could directly affect vascular permeability (Kurane 1997); epitopes from dengue virus might mimic host proteins, resulting in excessive tissue damage; or more complex interactions between virus strains and serotypes could result in altered peptide-ligand antagonism, impairing protective virus-specific T cell responses (Klennerman, Rowland-Jones et al. 1994).

In order to understand these possibilities during natural dengue virus infections we would need to study CD8<sup>+</sup> T cell cellular immune responses and serotype cross-reactivity as a result of natural dengue infection. Therefore, the aim of this chapter was to investigate dengue-specific and cross – reactivity CD8<sup>+</sup> T Lymphocyte responses during acute disease and convalescence, in a population of naturally immune individuals, in a dengue-endemic region.

#### **4.2 Methodology for this study.**

Naive Cytotoxic T-Lymphocytes (CTLs) are initially activated when their T-cell receptors (TCR) strongly interacts with surface class I HLA molecules on the target cells which have bound peptides from the pathogen or tumour antigens. Once activated, the CTL undergoes a process called clonal expansion in which it gains functionality, and divides rapidly, to produce an army of “armed”-effector cells and then travel throughout the body in search of cells bearing that unique MHC Class I – peptide complex. When exposed to these infected, effector CTLs release of cytokines such as IFN $\gamma$  and TNF $\alpha$ , chemokines such as RANTES and MIP-1 $\alpha$ , and causing the lysis of the target cell. Upon resolution of the infection, most of the effector cells will die and be cleared away by phagocytes, but a few of these cells will be retained as memory cells. Upon a later encounter with the same antigen, these memory cells quickly differentiate into effector cells, dramatically shortening the time required to mount an effective response. Therefore, in most studies of human CTLs, the aim is to detect and expand in vitro this memory-cell population from peripheral blood mononuclear cells (PBMCs).

CTLs recognize small peptides (of between 8 and 11 amino acids in length) from foreign proteins that have been broken down in the cytosol of infected cells. Peptide fragments are translocated to the endoplasmic reticulum and bind to newly synthesized class I molecules in a groove formed by the alpha helices of the  $\alpha 1$  and  $\alpha 2$  domains. Human HLA molecules are highly polymorphic and most of the polymorphism occurs in the peptide-binding groove. Therefore, different HLA molecules bind different kinds of peptides and a key component of CTL studies is to identify the dominant peptide targets of CTL recognition associated with individual class I HLA molecules for a particular pathogen.

Although with limited sample size (309 dengue cases) and with not ideal controls (251 “healthy” control), results from a case-control study of Vietnamese patients with DHF possibly suggests that variation at the HLA-A locus was associated with susceptibility to DHF and class I restricted cellular immune responses have an important influence on susceptibility to DHF (Loke, Bethell et al. 2001). HLA-A\*11 is one of the most common class I alleles in worldwide populations, ranging from 4 to 33% depending on the particular ethnic background (Sidney, Grey et al. 1996), and is highly prevalent in Southeast Asia. Fifteen natural variants (-A\*1101 to -A\*1115) have been reported to date, with HLA-A\*1101 being most prevalent. We chose to study HLA-A11, as it is one of the most common alleles in the Vietnamese population.

Major Histocompatibility Complex molecules associated with peptides derived from endogenous or exogenous proteins serve as ligands for TCR. The MHC/TCR

interaction is often viewed as a specific process, which involves one given TCR and only one MHC:peptide combination.

The allele-specific interaction usually involves a sequence of nine to eleven amino acids spanning the MHC groove. For class I molecules, the entire peptide ligand is involved in allele-specific interaction with MHC but for class II, the peptides are longer and the nine amino acid sequence is roughly central to the peptide. Allele-specific interactions are brought about by anchoring peptide side chains in complementary pockets in the MHC groove. The combination of allele-specific peptide-MHC interaction requirements can be described as a “peptide motif”, characterized by number, spacing and specificities of anchors, as well as the more degenerate preferences at non-anchor positions within the nonamer stretches. (Rammensee 1995).

Target cells are able to present 15-20mer peptides to specific CTLs, although the exact method of processing these longer peptides to the optimal 8-11 amino acid epitope remains obscure.

In theory, there are two approaching options for epitope identification. The panels of overlapping peptides of 15-20 amino acids representing the sequence of the target protein can be used to map the approximate region recognized by a CTLs. The optimal epitope can then be precisely defined using truncated peptides. The other approach is selection of 8-11mer peptides guided by knowledge of the peptide-binding 'motif' of the restricting class I molecule (Hill, Elvin et al. 1992) (Rowland-Jones, Sutton et al. 1995) or using more detailed computer algorithms.

In this present study, panels of 9-10mer predicted peptides were listed by using peptide- binding motif in combination with computer algorithms.

The peptide-binding motif of HLA-A\*1101 is defined by a preference for residues with hydrophobic amino acid in position 2 (valine, threonine, or isoleucine) and with positively charged side chains (Lysine or Arginine) at the C terminus position (position 9 or 10) (Falk, Rotzschke et al. 1994) (Kubo, Sette et al. 1994) (Zhang, Gavioli et al. 1993).

Table 4.1: HLA-A\*1101 motifs (Davenport MP 1996)

	Amino-acid position									
	1	2	3	4	5	6	7	8	9	10
HLA-A*1101		V/I/T							K	K
	(F/Y/M/A)		(M/L/F/I/Y/A)				(L/I/Y/V/I)		(R)	(R)

In order to meet the needs of scientists involved with MHC-associated peptides and to provide a handy source of MHC ligands and epitopes and related entries, a number of prediction servers are available over the World Wide Web such as BIMAS ([http://thr.cit.nih.gov/molbio/hla\\_bind/](http://thr.cit.nih.gov/molbio/hla_bind/)), SYFPEITHI (<http://www.syfpeithi.de/>), etc. Therefore, the possibility of epitope predictions for a limited number of MHC motifs has been opened. Theoretical predictions potentially save time and money. Instead of synthesizing and testing hundreds or even thousand of peptides, a preselection of a small set of peptides is made. The sequence of the protein or its gene, the restriction element, and its respective motif has to be available. Motif-based predictions result in a list of peptides that have a high probability of being presented by MHC class I molecules. However,

theoretical approaches cannot always guarantee success because not all details of the motifs are entirely known. The occurrence of unusual anchor amino acids can be compensated by refining the motif, but still a small percentage of epitopes do not act in accordance with any rules of the respective motif. In these cases, the theoretical approach will fail and experimental epitope mapping is unavoidable (Rammensee 1999).

### **4.3 Peptide panel of epitope prediction**

Peptides that were selected from the amino-acid sequence of dengue virus serotype 2 (DV2) isolate (strain 16681) based on published peptide-binding motifs for HLA-A\*11 (Lysine or Arginine at the C terminus position) and by using SYFPEITHI website, an HLA peptide binding prediction offered by the National Institute of Health – USA. Dengue serotype 2 virus (D2V) has been circulating and dominated over last few years in Viet Nam (Pasteur Institute of HCMC data). The D2V strain 16681 sequence was chosen because it is well-characterised, and is widely used in laboratory studies. Moreover, the strain 16681 is closely related to the circulating D2V strains in Viet Nam (Twiddy, Farrar et al. 2002).

A total of 61 peptides were synthesized by standard, solid phase Fmoc chemistry at the Weatherall Institute of Molecular Medicine, Oxford UK using a semi-automated peptide synthesizer (Zinnser Analytical, Germany). The purity ranged between 30-90% as determined by high performance liquid chromatography and the concentrations were estimated using BCA protein assay kit. These 61 peptides (28 nonamers and 33 decamers) corresponded to the amino acid sequence of the capsid (1 peptide), preM (1 peptide), Env (13 peptides), NS1 (7 peptides), NS2 (4



peptides), NS3 (10 peptides) and NS4 (6 peptides) viral antigens from D2V. (See Appendix 1)

They were arranged into 19 peptide pools and constructed a matrix of peptides. and the list is as below:

<b><u>Pool 1 to 12:</u></b>	5 – 6 peptides in each pool (less or equal 1 NS3 peptide/pool)
<b><u>Pool 13 – 14:</u></b>	NS3 peptides (5 peptides in each pool)
<b><u>Pool 15:</u></b>	C + preM + NS1 (9 peptides/pool: 1 C, 1 preM, 7 NS1)
<b><u>Pool 16:</u></b>	E (13 peptides/pool)
<b><u>Pool 17:</u></b>	NS2 + NS4 (10 peptides/pool)
<b><u>Pool 18:</u></b>	NS5 (10 peptides of 9mers/pool)
<b><u>Pool 19:</u></b>	NS5 (9 peptides of 10mers/pool)

In this matrix arrangement, each peptide is present in two different pools. This allows rapid identification of the peptide within a pool that is responsible for evoking a response in the IFN- $\gamma$  ELISPOT assay, since this peptide should drive a response in two different pools. PBMC were initially tested against 19 different peptide pools. The peptides from pools that evoked an IFN- $\gamma$  response were subsequently tested individually using IFN- $\gamma$  enzyme-linked immunospot assay (ELISPOT) (see Chapter 2).

In our present study, ELISpot assays were used to screen for peptide-specific responses and to quantify these in PBMCs from patients with dengue (this chapter). Later, tetramer assays and CTL lysis assays will be used for further study on functional phenotype of CD8<sup>+</sup> CTLs, CTL lines and clones (chapter 5) against relevant immunodominant peptide(s) discovered from this current works.

## **4.4 Results**

### **4.4.1 Patient population**

Thirty-one HLA-A\*11 patients with laboratory-confirmed dengue of varying clinical disease severity, attending the Hospital for Tropical Diseases in Ho Chi Minh City, Viet Nam, from year 2002 to 2004 were recruited into this prospective study of cellular immune responses. Molecular HLA typing was performed by Tim Rostron from WIMM Oxford using amplification refractory mutation system PCR (ARMS-PCR) with sequence specific primers, as described previously (Bunce, O'Neill et al. 1995). Written informed consent was obtained from the patient. The study protocol was approved by the Scientific and Ethical Committee at The Hospital for Tropical Disease and Oxford Tropical Research Ethical Committee (OXTREC) UK. Approximately 10 mL of venous blood samples were collected between 8 and 11am on the first morning after admission (study day 1), and again on study day 5 unless the patient was discharged earlier. Convalescent samples were obtained at 2 weeks and 1 month post-admission. The mean age was 21 years (range 15-31 years) and the mean length of illness before admission was 5 days (SD, 1 day, range 3 - 6 days). Serology on paired plasma samples indicated all patients were experiencing a secondary dengue infection. Among 31 patients, 12 patients had DSS, 19 patients had DHF grade I or II. There were 4 patients with DEN-2, 3 with DEN-4, 1 with DEN- and 1 with mixed DEN-1 & DEN-4 infections. DEN-3 was not be detected by RT-PCR in this group of patients. It was not possible to identify the infecting serotype by RT-PCR in the remaining 22 patients, presumably because they had already resolved their viraemia when

admitted. The average haematocrit on hospital admission was 45% (SD, 5.1%, range, 38 -57%), the mean of the platelet count was 50,903 (/mm<sup>3</sup>) (SD, 40,519; range 14,000-155,000) and the mean of the white blood cells was 4,784 (/mm<sup>3</sup>) (SD 1,831; range 1,400-8,540). (Appendix 2)

#### **4.4.2. IFN $\gamma$ ELISPOT responses against a panel of peptides**

PBMCs from convalescence samples (3 – 4 weeks after hospital admission) of those 31 adults experiencing a secondary dengue infection were assayed by IFN- $\gamma$  ELISPOT. The number of spot-forming units (SFU) in each well was counted and was subtracted the background (no antigen stimulation). The range of spots in negative control was less than 20 (SFU)/million PBMCs. The criteria for a positive ELISPOT are  $\geq 100$  spot forming units (SFU)/million PBMCs.

In 14 patients (45%), one or more individual peptides reproducibly evoked an IFN- $\gamma$  response in the ELISPOT assay.

In total, 6 different peptides were recognized by T cells from 14 patients. A summary of the peptides contained potentially novel T-cell epitopes that evoked responses in the IFN- $\gamma$  ELISPOT assay is provided in Table 4.2. The magnitude of the response varied by patient and by peptide. Of 14 patients who had ELISPOT responses, DEN-2 was detected by RT-PCR in 3, DEN-4 in 3 patients.

There were nine patients who made responses to one peptide and five patients who made response to two or more individual peptides (Figure 4.1).

Table 4.2: List of peptides that evoked responses in IFN- $\gamma$  ELISPOT assays.

D2V Peptides	Sequence	Magnitude (SFU/10 <sup>6</sup> PBMCs)		Number of patients (14 positive / 31 screened pts)						Note
		Average	Range	Total (31)	D1 (1)	D2 (4)	D3 (0)	D4 (4)	Unknown (22*)	
Env <sub>226-234</sub>	TQGSNWIQK	850	100 - 1705	6	0	1	0	2	3	
NS1 <sub>161 - 170</sub>	GVFTTNIWLR	186	100 – 506	9	0	2	0	3	4	
NS3 <sub>52 - 61</sub>	VTRGAVLMHK	435	n/a	1	0	1	0	0	0	
NS3 <sub>133 – 142</sub>	GTSGSPIIDK	384	109 - 1005	8	1*	1	0	3*	4	*One DV1 & DV4 co-infection
NS4 <sub>12 - 20</sub>	RLPTFMTQK	296	215 – 377	2	0	0	0	2	0	
NS5 <sub>362 - 371</sub>	RTQEPKEGTK	760	n/a	1	0	0	0	0	1	

Figure 4.1: Frequency of patients that their PBMCs made responses to one or more individual HLA-A\*11 dengue 2 serotype peptides.

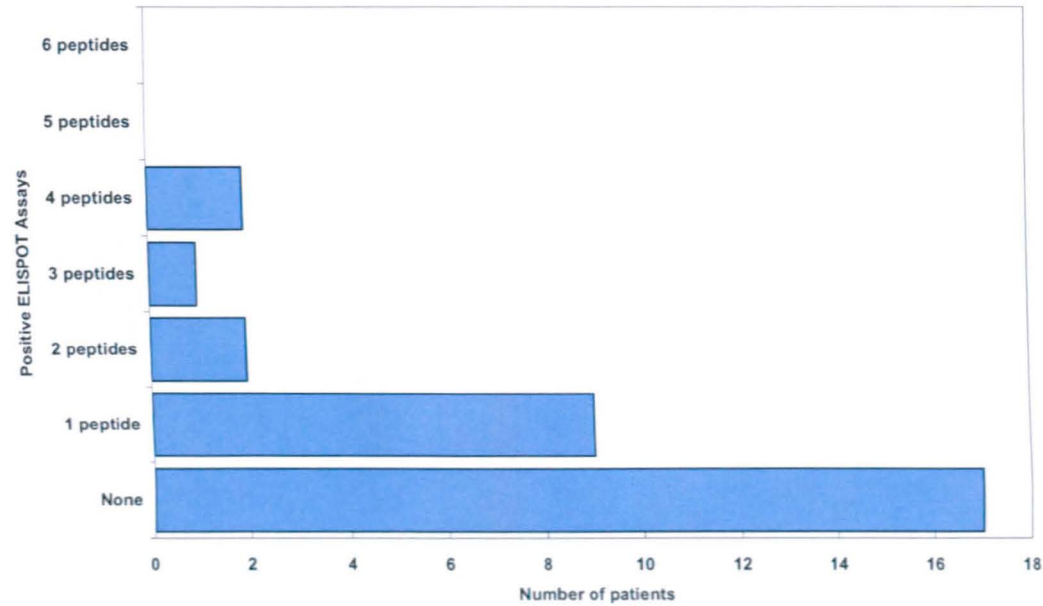
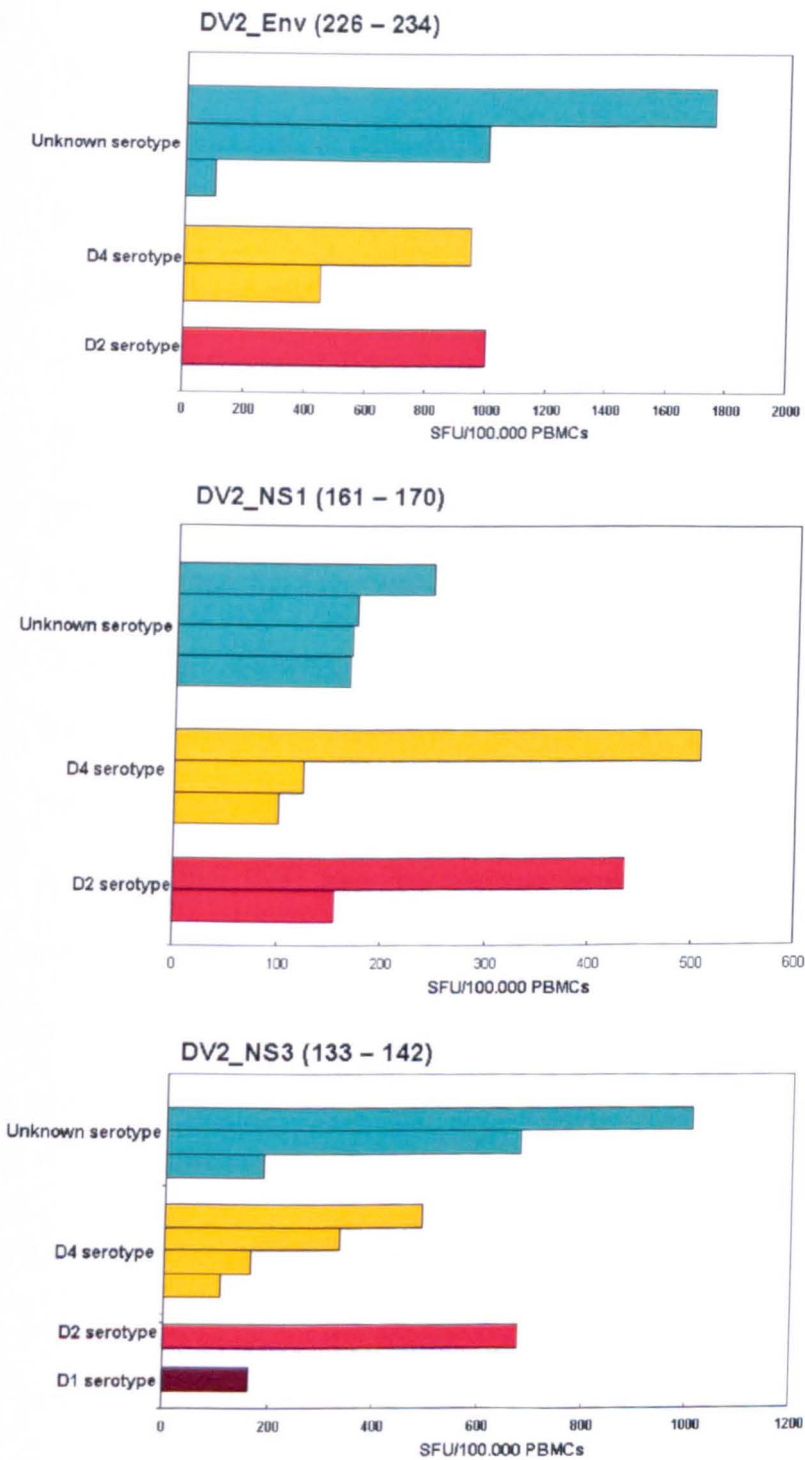


Figure 4.2: Magnitude of T cell responses from dengue infected patients to HLA-A\*11 dengue 2 serotype peptides.



Interestingly, all of four DEN-4 infected patients (including 1 patient with mixed DEN-1&DEN-4 infection) and 3 among four DEN-2 infected patients made responses to one or more individual HLA-A\*11 DEN-2 peptides. The magnitude of T cell responses to peptide Env<sub>226-234</sub>, NS1<sub>161 - 170</sub>, and NS3<sub>133 - 142</sub> in DEN-2 infected patients varied amongst individuals. Responses against these same peptides in patients infected with an unknown serotype are also presented for comparison (Figure 4.2).

At the time this work was conducted, other scientists at the Wetherall Institute of Molecular Medicine, Oxford had identified NS3<sub>133 - 142</sub> as dominant dengue CD8+ T cell epitope by using an overlapping peptide approach in ELISPOT assays. Peptide-HLA tetrameric complexes of this NS3<sub>133 - 142</sub> peptide have been also folded successfully. Moreover, NS3<sub>133 - 142</sub> peptide was the only peptide recognized by subjects infected with all three serotypes identified in our study, an indication that there may be serotype cross-recognition. Given the strength of evidence implicating NS3<sub>133 - 142</sub> as a T cell epitope, we decided to focus on the immune response of CD8+ T cells of this epitope in term of specificity and cross-reactivity between the four dengue serotypes.

#### **4.4.3. Cross reactivity of the CD8+ T cell response to NS3<sub>133 - 142</sub>**

Sequence analysis of the decamer NS3<sub>133 - 142</sub> suggested there were 4 major variants among the published dengue NS3 sequences (Table 4.3).

Table 4.3: Main variants of Dengue *NS3*<sub>133-142</sub> peptides.

Peptide	Serotype of dengue virus	Amino acid sequences
pD1	DEN-1	GTSGSPIVNR
pD2.1	DEN-2	GTSGSPIIDK
pD2.2	DEN-2	GTSGSPIVDK
pD3/4	DEN-3 and 4	GTSGSPIINR

The *NS3*<sub>133-142</sub> sequence in pD1 (GTSGSPIVNR) differed by three amino acids from previous predicted pD2 peptide (GTSGSPIIDK). The *NS3*<sub>133-142</sub> sequence in DEN-3 and DEN-4 are identical (GTSGSPIINR) and they differed by one amino acid from pD1 (Isoleucine in replaced for Valine at position 8) and two last amino acids from C-terminal anchor from previous predicted pD2 peptide. There is also an other variant of *NS3*<sub>133-142</sub> sequence in DEN-2 that differed from previous predicted pD2 peptide by 1 amino acid at position 8 (Valine was replaced by Isoleucine) and named as variant pD2.2 (GTSGSPIVDK).

Acute phase PBMCs (day 6 – 7 of the illness) of 15 adults experiencing a secondary dengue infection with known dengue serotypes from prospective study of cellular immune responses were tested by IFN $\gamma$  ELISPOT assays against 4 variants of *NS3*<sub>133-142</sub> decamers. In those 15 patients, there were 3 donors with dengue serotype 1 infection (20%), 7 donors with dengue serotype 2 infection (47%), 1 donor with dengue serotype 3 infection (7%) and 4 donors with dengue serotype 4 infection (26%) (see Appendix 3).



The magnitude of IFN $\gamma$  ELISPOT assays responses varied by patient, by serotype and by variant of NS3<sub>133-142</sub> peptide as showed in Table 4.4. (The number of spot-forming units in each well was counted and was subtracted the background).

There were few differences between ELISPOT responses to pD1 and pD3/4 in each individual with DEN-1 or DEN-3, 4 infections. However, in those groups of patients, responses to the pD2.1 were weaker, and particularly, responses to pD2.2 variants were comparably low (in BC395, DF110 and BC428) or even negative.

Interestingly, the magnitude of CD8<sup>+</sup> T cell responses to cognate peptide variants corresponding to the current infecting DEN-2 infected patients (i.e. pD2.1 and pD2.2 against DEN-2 patients) were significantly weaker compared with those in patients infected by other serotypes (i.e. pD1 in DEN-1 and pD3, 4 in DEN-3/4 infections respectively). Moreover, except patient DF129 who had positive response to pD1 and pD3/4, ELISPOT responses to pD1 and pD3/4 in patients with dengue serotype 2 infection were all negative.

Table 4.4: IFN $\gamma$  ELISPOT assays of acute phase PBMCs (day 6 – 7 of illness) with different serotype infection againsts variants of Dengue NS3<sub>133-142</sub> peptides.

Patients	Dengue Serotype	RESULTS (SFU/10 <sup>6</sup> PBMCs)*			
		DV1_NS3 <sub>133-142</sub>	DV2.1_NS3 <sub>133-142</sub>	DV2.2_NS3 <sub>133-142</sub>	DV3/4_NS3 <sub>133-142</sub>
BC337	1	Positive (2380)	Positive (1340)	Negative (44)	Positive (2112)
BC354	1	Positive (600)	Positive (117)	Negative (42)	Positive (400)
BC392	1	Positive (420)	Negative (90)	Negative (73)	Positive (243)
BC338	2	Negative (0)	Negative (0)	Negative (40)	Negative (0)
BC378	2	Negative (0)	Negative (15)	Negative (55)	Negative (30)
BC393	2	Negative (0)	Negative (0)	Negative (41)	Negative (0)
BC395	2	Negative (4)	Negative (12)	Positive (164)	Negative (0)
BC514	2	Negative (8)	Negative (8)	Negative (8)	Negative (0)
DF110	2	Negative (0)	Negative (0)	Positive (185)	Negative (0)
DF129	2	Positive (152)	Positive (332)	Negative (24)	Positive (200)
DF141	3	Positive (1376)	Positive (828)	Negative (56)	Positive (1432)
BC383	4	Positive (1240)	Positive (616)	Negative (44)	Positive (1268)
BC390	4	Positive (1665)	Positive (550)	Negative (30)	Positive (1655)
BC428	4	Positive (1708)	Positive (500)	Positive (108)	Positive (1662)
DF135	4	Positive (2080)	Positive (990)	Negative (75)	Positive (2240)

\* The number of spot-forming units (SFU) in each well was counted and was subtracted the background (no antigen stimulation). The range of spots in negative control was less than 20 (SFU)/million PBMCs. The criteria for a positive ELISpot are  $\geq 100$  spot forming units (SFU)/million PBMCs.

#### 4.4.4 Ex – vivo cell surface staining for PBMC with NS3<sub>133 – 142</sub>

As ELISpot does not distinguish between responses to the individual serotype peptides of the same population of T cells in term of the cross reactivity, we went on to perform tetramer cell surface staining.

Acute and convalescent PBMC from 31 HLA-A\*1101 positive dengue patients were re-screened for responses to the previously defined HLA-A11 dengue NS3<sub>133-</sub>

142 epitope using tetramer staining with a panel of peptides representing the main serotype variants of this epitope. In this study, for pD2, we concentrated on variants 2.1 as it seems make more responses than variant 2.2 (results from Table 4.4). Summary of three variants of HLA-A11 dengue NS3<sub>133-142</sub> epitope is described in Table 4.5

Table 4.5: Panel of peptides from dengue NS3<sub>133-142</sub> epitope using in tetramer study.

Peptide tetramers	Serotype	Amino acid sequences
pD1	Dengue 1	<b>GTSGSPIVNR</b>
pD2	Dengue 2	<b>GTSGSPIIDK</b>
pD3/4	Dengue 3 and 4	<b>GTSGSPIINR</b>

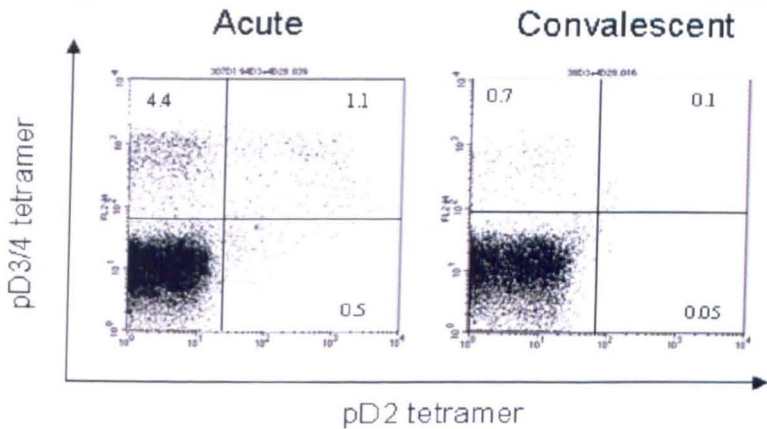
The HLA-A\*1101 tetramers assembled with NS3<sub>133-142</sub> peptides from different serotypes were conjugated with different fluorochromes in order to be able to examine the ability of CD8<sup>+</sup> T-cells to recognize more than one dengue variant by co-staining of T-cell populations. Cell surface staining was carried out on carefully thawed cryo-preserved PBMCs. Titrated tetramers (PE conjugated or quantum-red conjugated) were added for 15 min at 37°C, then the cells were incubated with Anti-CD8-APC and anti-CD38-FITC antibodies (Becton Dickinson - San Diego, California) for 15 min at room temperature (RT). Cells were then washed and stored in Cell Fix<sup>TM</sup> buffer (Becton Dickinson) at 4°C until flow cytometry analysis was performed. Samples were analyzed on a Becton Dickinson FACSCalibur (Chapter 2).

Although one adult DHF grade II patient (BC307), a 17 year old woman admitted on day 6 of illness with confirmed secondary acute dengue infection (unknown serotype) had HLA-A11 restricted NS3<sub>133-142</sub> -specific responses detected in both the acute and convalescent samples were selected for this study. The rest majority of patients showed much less significant staining.

This patient had a high frequency of ex-vivo tetramer positive populations in both acute and convalescent samples allowing direct analysis without the need for in-vitro expansion. In contrast, the majority of the rest of HLA-A\*1101 positive dengue patients were screened by tetramer staining showed much less significant staining.

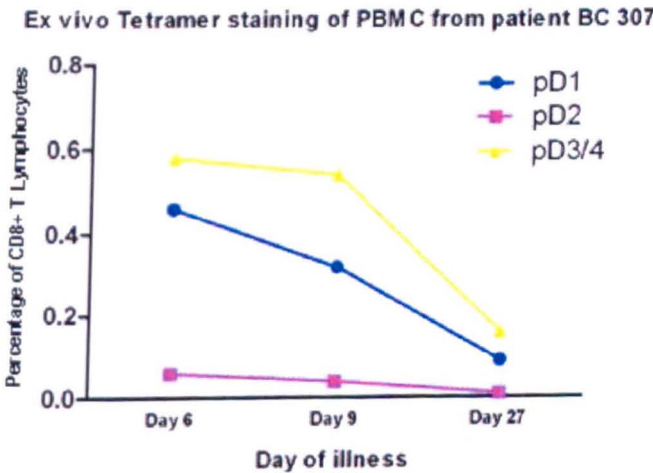
In-patient BC307, approximately 18% of NS3<sub>133-142</sub> -tetramer positive cells in acute PBMC were cross-reactive, recognizing both the pD2 and pD3/4 tetramers; 73% were pD3/4 specific and 9% were pD2 specific. The cross - reactive population was not detectable in the convalescent (day 27) sample (Figure 4.3). The frequency of tetramer positive CD8<sup>+</sup> T lymphocytes from PBMC samples incrementally declined from day 6 to day 9 and day 27. CD8<sup>+</sup> T cell populations binding the pD1 and pD3/4 tetramers were present in higher frequencies compared with T cells binding pD2 tetramer (Figure 4.4).

Figure 4.3: Ex vivo pD2 and pD3/4 tetramer staining of PBMC from patient BC3071



Freshly thawed cryopreserved PBMCs from BC307 were stained with A11 pD3/4 and pD2 tetramers and CD8 antibody. The plots are gated on CD8 positive lymphocytes. The majority of cells in the acute sample (left) are specific for pD3/4 but there is a significant population cross-reactive with this and pD2. The convalescent sample (right) shows only pD3/4 specific cells.

Figure 4.4: Frequency of Dengue specific CTL from PBMC on acute and convalescent phase.



Freshly thawed cryopreserved PBMCs from BC307 on Day 6, Day 9 and Day 21 of illness were stained with A11-pD1, pD2 and pD3/4 tetramers and CD8 antibody. The plots are gated on CD8 positive lymphocytes

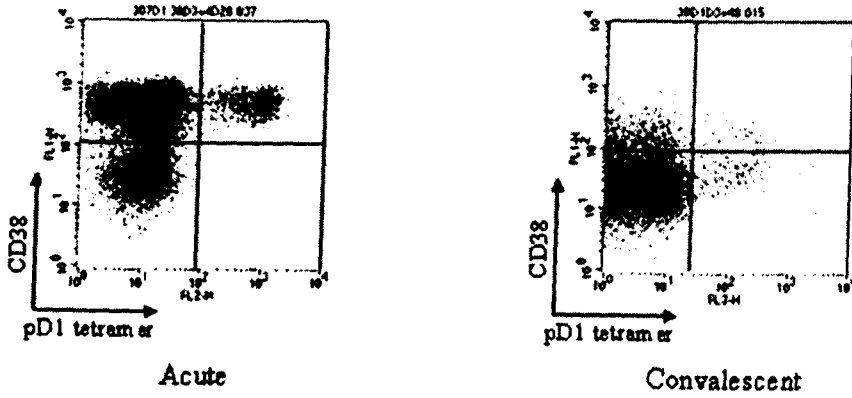


CD38<sup>+</sup> is a marker of lymphocyte activation. *Ex vivo* staining of PBMC from patient BC307 demonstrated equally high levels of CD38 staining among all tetramer positive cells in the acute phase. By three weeks after the acute sample CD38 expression had fallen markedly (Figure 4.5.a). At this time point CD8 cells recognizing the two similar peptide variants, pD1 and pD3/4, were present (Fig 4.5.b). Interestingly, there were relatively different levels of binding to the pD1 and pD3/4 tetramers, with a population of cells showing high cross-reactivity to the two tetramers (gate R7 in Fig. 4.5.b) and second population showing relatively less cross-reactivity (gate R6 in Fig. 4.5.b).

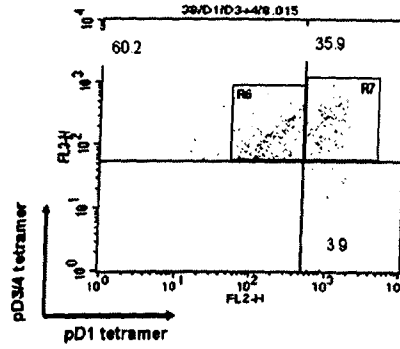
CD38 expression was greater on those T cells with the highest level of cross-reactivity to the pD1 and pD3/4 tetramers (57% CD38 high – Figure 4.5.c - Right) compared to cells with relatively less cross-reactivity to the pD1 and pD3/4 tetramers (19% CD38 high – Figure 4.5.c - Left). This suggests that highly cross-reactive T cell populations are more activated than cells exhibiting low levels of cross-reactivity.

**Figure 4.5: Highly cross-reactive CTL are more activated than partially cross-reactive CTL.**

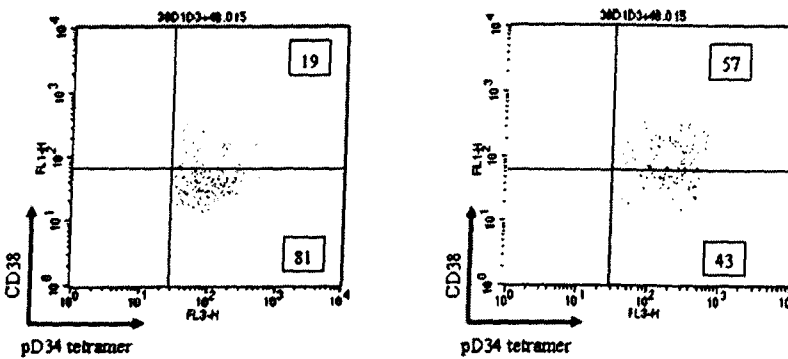
(a)



(b)



(c)



(a) Frozen PBMC from patient BC307 on acute and day 21 of illness were. The cells showed in the plot were gated on tetramer positive CD8+ cells.

(b)&(c) Convalescent sample from this patient were co-stained with CD38 (FL1), pD1 (FL2), pD3/4 (FL3) tetramers and CD8. (b) Lymphocytes gated on tetramer positive CD8+ cells. Cross-reactivity population with pD1 and pD3/4 tetramers were divided into 2 sub-groups. (c) Highly cross-reactive cells (Gate R7) show higher levels of expression of CD38 (right) than partially cross-reactive cells (Gate R6) (left).

## **4.5 Discussion**

### **4.5.1 Epitope prediction using IFN $\gamma$ ELISPOT with predicted peptides**

T cell responses to viral pathogens provide important direct and indirect assistance in the control of viral replication and viral eradication. In the context of dengue, the breadth and specificity of in vivo T cell responses to dengue viral antigens remains relatively poorly characterized. Most studies of dengue-specific T cells have occurred in the context of T cell clones generated from live attenuated dengue virus vaccines, or less frequently, from dengue patients (Kurane, Brinton et al. 1991) (Green, Kurane et al. 1993) (Dharakul, Kurane et al. 1994) (Kurane, Okamoto et al. 1995) (Gagnon, Zeng et al. 1996) (Mathew, Kurane et al. 1996). Analysis of T-cell responses in patients after natural secondary infections is therefore important because it may provide insights into the mechanisms of T-cell-mediated immunopathology.

In this chapter, we sought to identify HLA-A\*11 restricted dengue-specific CD8+ T lymphocyte responses in a more direct fashion by combining the sensitive IFN- $\gamma$  ELISPOT assay with freshly collected PBMC from serologically- and virologically-defined natural secondary dengue cases using motif-based predicted peptides. This approach has the advantages of speed, simplicity and the avoidance of artifacts generated by lengthy in vitro restimulation.

With the screening over 31 patients experiencing a secondary dengue infection, we identified 6 peptides that could evoke T cell responses in interferon-gamma ELISPOT assay. D2V\_NS1<sub>161-170</sub> epitope is recognized by 2 out of 4 DEN2- and by 3 out of 4 DEN4-infected patients; Env<sub>226-234</sub> peptide is also recognized by 6



patients. Further work will be required to determine if all of these peptides are genuine HLA-A\*11 restricted CD8+ T cell epitopes.

However, multiple lines of evidence indicate that the DV2 NS3<sub>133-142</sub> peptide (GTSGSPIIDK) is indeed a HLA-A\*11 restricted CD8+ T cell epitope. First, in this present study we demonstrated that the NS3<sub>133-142</sub> peptide could be folded into a MHC-class I complex tetramer and that this could be used to stain specific CD8 T cell populations in PBMC of dengue patients. Secondly, Mongkolsapaya *et al*, using an overlapping peptide approach then a set of truncated peptides, also identified NS3<sub>133-142</sub> as a CD8+ T cell epitope restricted through HLA-A\*11 (Mongkolsapaya, Dejnirattisai et al. 2003). Mongkolsapaya *et al* found that the optimal peptide for the NS3<sub>133-142</sub> serotype 2 variant to be the 11-mer peptide GTSGSPIIDKK, although there were also responses to the 10-mer peptide GTSGSPIIDK, truncated by one amino acid at the C terminus. However, 10-mers were optimal for the NS3<sub>133-142</sub> serotype 1, 2 and 3 variants than 11-mers (extra C-terminal amino acid), suggesting they were using the arginine at position 10 as the C-terminal anchor.

The NS3<sub>133-142</sub> epitope is one of several epitopes located within the NS3 protein. The importance of NS3 as a target of T cell responses has been demonstrated in parallel studies at the Hospital for Tropical Diseases (Simmons, Dong et al. 2005). These studies identified thirty-four peptides containing potentially novel T-cell epitopes, with peptides from NS3 being over-represented. These results highlight the importance of NS3 and T cells during acute secondary infection.

#### 4.5.2 Cross reactivity of predicted peptides

After acute dengue infection, PBMCs from convalescence samples (3 - 4 weeks after admission) were able to be stimulated by DEN-2 A\*11 restricted peptides by ELISPOT assays. The magnitudes of responses varied by patient and by peptide and there was cross-reactive responding of PBMCs from non-DEN-2 infected patients to pD2 peptide stimulation.

There are 4 variants of the sequence of Env<sub>226-234</sub> when performing sequence analysis: TQGSNWIQK of D2V; TKTPTWNRK of D3V, TSQETWNRQ of D1V and TSEVHWNYK of D4V. In this study, the D2V\_Env<sub>226-234</sub> peptide could stimulate ELISPOT responses in convalescence PBMCs from two DEN-4 infected patients and one DEN-2 infected patients. Therefore, further analysis with all 4 variants of this suspected epitope is needed for confirmation the cross-reactivity of this epitope.

In term of cross-reactivity of NS<sub>3133-142</sub> epitope variants, NS<sub>3133-142</sub> pD2 was recognised by convalescent PBMCs (3 – 4 weeks after hospital admission) from one DEN-1, one DEN-2 and four DEN-4 infected patients. Interestingly, PBMCs from acute phase (day 6 – 7 of the illness) in either DEN-1 or DEN-3 or -4 infection showed relatively strong responses to NS<sub>3133-142</sub> pD1 and NS<sub>3133-142</sub> pD3/4 and weaker responses to pD2. These results could possibly be explained by the high sequence similarity in peptide sequence between NS<sub>3133-142</sub> pD1 and NS<sub>3133-142</sub> pD3/4 (only one amino acid at position 8 - Valine was replaced by Isoleucine) and the different of 2 or 3 amino acid in those respectively peptide sequences versus NS<sub>3133-142</sub> pD2.

In our study, in convalescent phase (3 – 4 weeks after hospital admission), the NS3<sub>133-142</sub> pD2 gave strong responses in PBMCs of DEN-2 infected (serotype - specific) and from DEN-4 infected patients (cross – reactive) (see Tab 4.2 and Fig 4.2); in acute phase (day 6 – 7 of illness), responses to pD2 variants (including pD2.1 and pD2.2) are weak or negative in patients with DEN-2 (serotype - specific) but stronger in patients with Dengue 1, 3 or 4 infections (cross – reactive) (see Table 4.4). There were three out of seven DEN-2 infected patients reacted to pD2 in acute phase, among those, only one patient (DF129) showed cross-reactivity against pD1 and pD3/4 with this was of low magnitude. In contrast, although only one of four DEN-2 infected samples from the convalescent phase responded to pD2.1, the response magnitude was high.

Mongkolsapaya *et al* found low or absent ELISpot responses against those HLA-A11\_NS3<sub>133-142</sub> peptides in samples taken during acute hospital admission and peak responses were observed at 2 weeks to 2 months follow-up (Mongkolsapaya, Dejnirattisai *et al.* 2003). Similar finding also reported in ELISpot responses against HLA-A24\_NS3<sub>556-564</sub> peptides (Mongkolsapaya, Duangchinda *et al.* 2006). In contrast to other virus infections such as HIV or Epstein – Barr virus (Appay, Dunbar *et al.* 2002), the absence of responses in acute secondary dengue 2 infection in our study and the low frequencies of antigen – specific T cells in Mongkolsapaya's studies could be explained by one of three possibility, or the combination of them. Firstly, these epitope specific cells may not have had time to proliferate fully at the time of sample was collected. Secondly, they may have been sequestered in peripheral tissues or in lymphoid organs. Lastly, these cells may

have been over-activated and the cells may have been dying at an increased rate due to activation – induced cell death (AICD).

Mongkolsapaya *et al* showed that there is a degree of cross-reactivity between the different epitopes. After secondary infection with DEN-1 or 3 (and 4) serotypes, in acute phase of currently DEN-2 infection, T cells reacted better with the HLA-A11\_NS3<sub>133-142</sub> pD1 and NS3<sub>133-142</sub> pD3 but lesser with NS3<sub>133-142</sub> pD2. The finding that secondary infection with a virus carrying a similar but distinct epitope can stimulate the proliferation of cross-reacting, low-affinity clones is interpreted as “original antigenic sin” phenomenon of T cells.

However, we could not generally reproduce this finding in our acute samples of 8 adult Vietnamese patients with DEN-1 and DEN-3, -4 infections. We speculated that an additional mechanism by which the order of sequential dengue serotype infections might influence phenotypic manifestations. In theory, a second dengue infection may occur in any of 12 possible sequences (1–2, 1–3, 1–4, etc.). Epidemiologic studies have suggested that the order of acquisition of dengue virus infections might be important. Classical DHF/DSS has occurred with infections in the sequence DEN-1 followed by DEN-2 (Guzman, Kouri et al. 1990), DEN-3–DEN-2, or DEN-4–DEN-2 (Sangkawibha, Rojanasuphot et al. 1984), and DEN-1–DEN-3 (Endy, Nisalak et al. 2004). Other sequences may also exist but have not been reported. Infections caused by the sequence DEN-1–DEN-3 were accompanied by DHF/DSS cases, whereas in the Americas from 1963 to 1977, many persons must have been infected in the sequence DEN-2–DEN-3 genotype V, but without developing DHF/DSS (Alvarez, Rodriguez-Roche et al. 2006).

Experimentally, by analyzing of murine CD8<sup>+</sup> T cell clones specific for the dengue virus NS3 protein, Spaulding found that the patterns of dengue virus serotype cross-reactivity in short-term T cell lines and clones differed in mice immunized with different dengue serotypes. CTL responses are influenced by the order of acquisition of serotypes of dengue virus (Spaulding, Kurane et al. 1999). In addition, Brehm, Welsh et al showed that previous viral infection markedly altered the establishment of virus specific memory CD8<sup>+</sup> T cells for subsequent infection with a heterologous virus encoding a cross-reactive peptide (Brehm, Pinto et al. 2002). Collectively these observations suggest that particular sequences of infection can have different biological phenotypes and this might extend to the characteristics of the acute T cell response to NS3<sub>133-142</sub> peptides.

#### **4.5.3 Ex – vivo cell surface staining for PBMCs & Over activation of cross-reactive CTL**

Although one adult patient (BC307) demonstrated a larger ex-vivo NS3<sub>133-142</sub> - specific tetramer positive population in acute samples, the rest (majority) of patients showed much less significant staining.

*Ex vivo* staining of PBMC from patient BC307 demonstrated equally high levels of CD38<sup>+</sup> staining among all tetramer positive cells in the acute phase. By three weeks after the acute sample CD38 expression had fallen markedly. At this time point CD8<sup>+</sup> cells recognizing the two similar peptide variants, pD1 and pD3/4, were present (partially cross-reactive) but cells cross-reactive between the more heterologous peptides, pD2 and pD3/4 could not be detected (highly cross-reactive cells). CD38 expression was greater on those T cells recognizing both pD1 and

pD3/4 (57%) than upon partially or non cross-reactive T cells (19%). This suggests that highly cross-reactive T cell populations are more activated than cells exhibiting low levels of cross-reactivity.

These observations, albeit limited to one patient, led us to the hypothesis that high levels of T cell activation in the acute phase of dengue are possibly associated with activation – induced cell death.

Others have found that tetramer staining of fresh blood does not always demonstrate large numbers of virus specific CD8 T cells in acute dengue infection (Mongkolsapaya, Dejnirattisai et al. 2003). As discussed in 4.5.2, this phenomenon may be a consequence of the timing of cell proliferation, the sequestration of T cells in infected peripheral tissues or in lymphoid organs, or the massive apoptosis of stimulated T cells or perhaps, the combination of them. The enhancement of viral replication caused by antibody-mediated immune enhancement may drive the T cells with highest affinity for the infecting virus into apoptosis, through the process of activation-induced cell death (Mongkolsapaya, Dejnirattisai et al. 2003). The over-activation of CD8<sup>+</sup> T Lymphocyte could possibly be associated with serotype cross – reactive T cells. Excessive immune activation may contribute to clinical disease during secondary infection. In particular, the rapid mobilization of serotype cross-reactive memory T cells that release vasodilator inflammatory molecules has been suggested to explain some aspects of the clinical syndrome (Kurane, Rothman et al. 1994). Relatively higher frequencies of activated, cross-reactive CD8<sup>+</sup> T cells (Zivna, Green et al. 2002; Mongkolsapaya, Dejnirattisai et al. 2003), and a range of direct and indirect markers of cellular immune activation

have been associated with severe disease during secondary infection (Kurane, Innis et al. 1991) (Kittigul, Temprom et al. 2000) (Green, Pichyangkul et al. 1999) (Green, Vaughn et al. 1999) (Green, Vaughn et al. 1999) (Gagnon, Mori et al. 2002) (Juffrie, Meer et al. 2001).

To gain further insight around this phenomenon, we generated CTL lines and clones stimulated with different variants of **NS3<sub>133-142</sub>** peptide from acute and convalescence PBMCs of secondary DHF patients then evaluated the functional phenotype of these cross-reactivity CD8<sup>+</sup> T cells (next chapter).

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**CHAPTER 5**

**FUNCTIONAL STUDY OF**

**CYTOTOXIC T LYMPHOCYTE**

**CLONES**

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## **5.1 Introduction**

In order to assess the fine specificities of dengue-specific CTL with limited cell numbers, and in the face of the possible down-regulation of cell-surface TcR expression in acute samples, we have studied further the specificity and cross reactivity of CD8<sup>+</sup> T-cells by generating short-term CD8<sup>+</sup> CTL lines and clones stimulated with the different variants of the immunodominant NS3<sub>133-142</sub> peptide (Table 4.5) from acute and convalescence PBMCs of DHF patients with secondary dengue. The functional phenotype of these cross-reactivity CD8<sup>+</sup> CTL lines and clones was then evaluated.

## **5.2 Results**

### **5.2.1 Highly cross-reactive CTL can be expanded from bulk culture of PBMC in acute infection but are less abundant in convalescence.**

PBMCs collected acutely (day 4-7 of illness) and during convalescence (3 months after illness onset) from three patients were used in this study. The details of the patients are summarized in (Table 5.1).

Acute and convalescence PBMC samples (5-10 x 10<sup>6</sup> cells/sample) from these patients were incubated with each of the dengue NS3<sub>133-142</sub> variant epitope peptides (Table 4.5) at 2 μM concentration for 1 hour, after which IL-7 (PeproTech) was added at 25 ng/ml. On day 3, 25 to 50 U of recombinant interleukin-2 was added. On day 14 and 20, CTL were double-stained with HLA-A11 tetramers assembled with different epitopes and labeled with different fluorophores (see chapter M&M).

Table 5.1: Acute secondary dengue infected patients had HLA-A11 restricted NS3<sub>133-142</sub> specific responses.

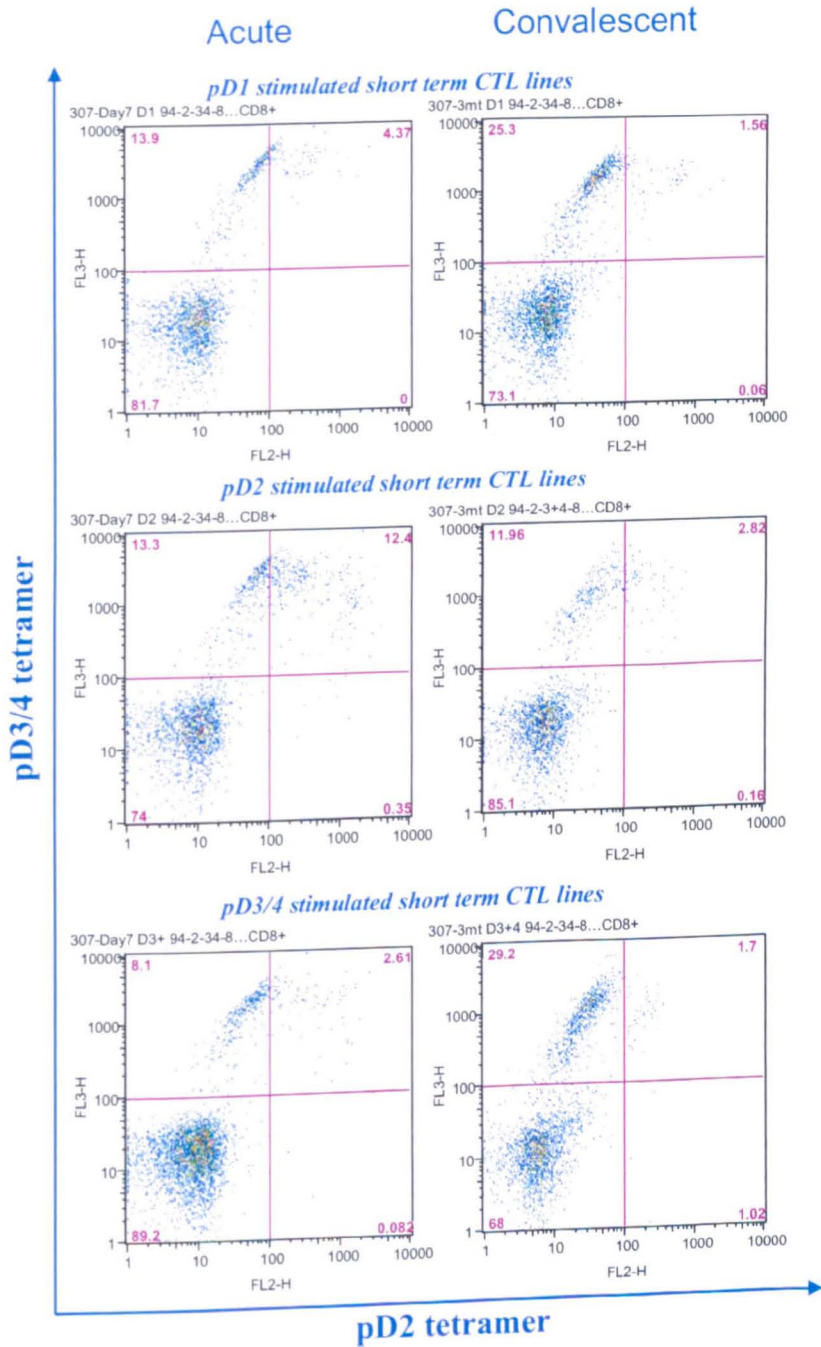
Patient ID	Age	Infecting serotype	Days of illness at the time of enrollment	Clinical Diagnosis
BC 307	17	Unknown	6	Secondary DHF III
MD 893	14	D4	3	Secondary DHF I
MD 856	11	D4	5	Secondary DHF I

We considered such T cell lines – those recognizing both pD2 and pD3/4 – to be highly cross-reactive given their equal recognition of both tetramers despite significant peptide sequence variation. T cell lines recognizing pD1 usually recognized pD3/4 – a reflection of their greater similarity in peptide epitope sequence.

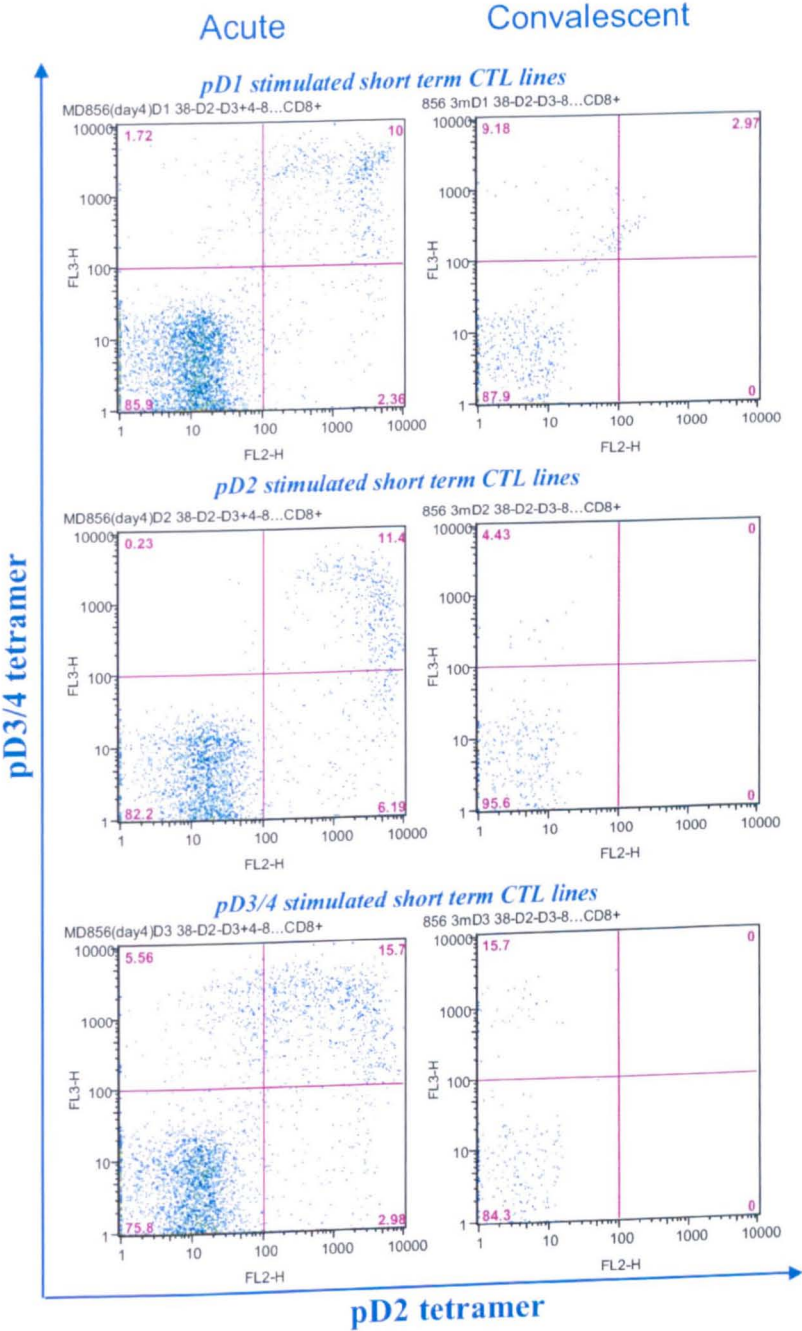
The pattern of staining differed slightly in each patients but all three patients demonstrated expansions of highly cross – reactive T cell lines (i.e. binding both pD2 and pD3/4 tetramers equally well) from acute samples after stimulation with each of the dengue NS3<sub>133-142</sub> variant epitope peptides (Fig. 5.1). In contrast to T cell lines generated from acute samples, those from convalescent samples did not contain the same proportion of T cells that were highly cross-reactive with the pD2 and pD3/4 tetramers (Figure 5.1). Serotype specific CTL lines (i.e. strong binding of the tetramer folded with the peptide of one serotype, weak binding of the other) were detected from acute and convalescent samples after stimulation with variants of epitope peptides.

Figure 5.1: Highly cross-reactive T cells can be expanded from acute but not convalescent PBMC from dengue-infected patients.

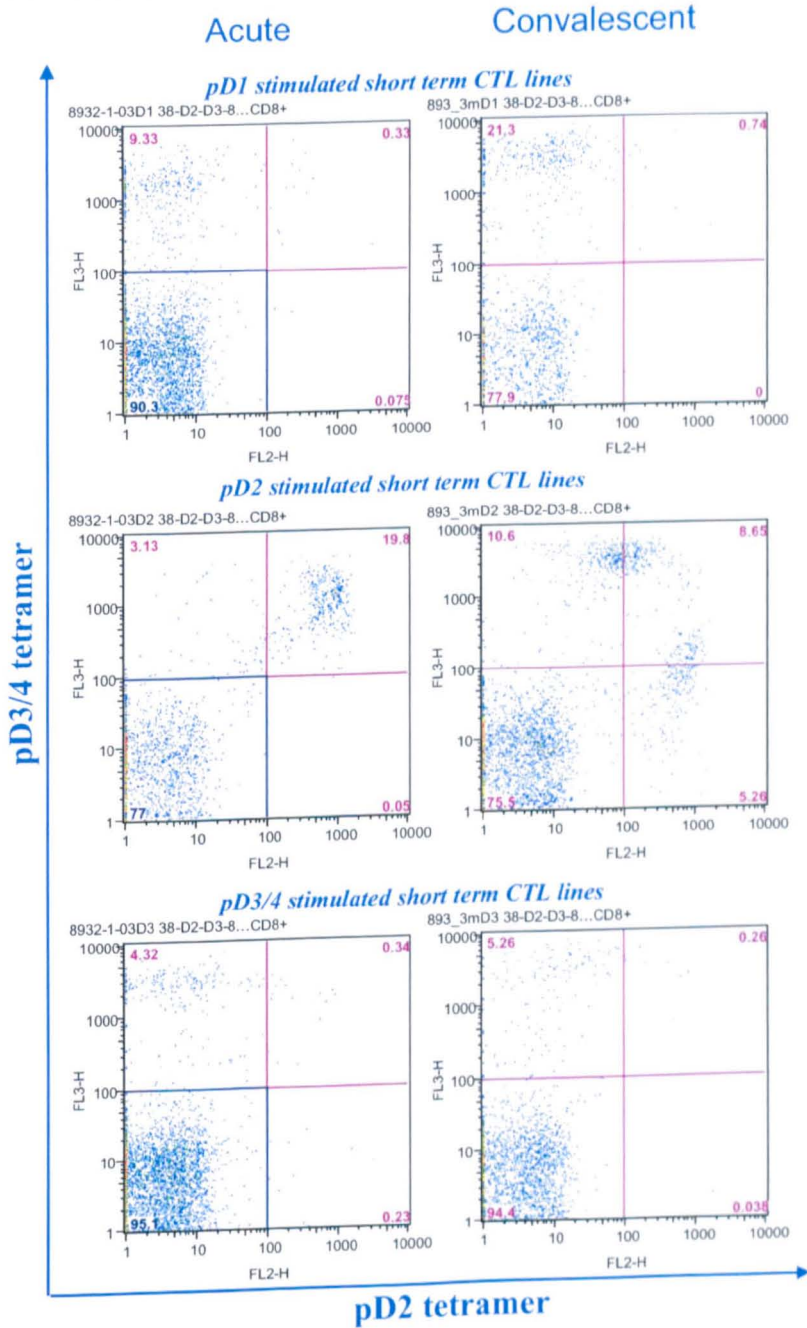
(a) Short term CTL lines from BC 307 PBMCs



(b) Short term CTL lines from MD856 PBMCs



## (c) Short term CTL lines from MD893 PBMCs



The left panel shows a CTL line grown from the acute sample, and the right panel shows CTL line grown from the convalescent sample. All lines were stained in parallel under the same conditions. Short term CTL lines were stimulated by pulsing PBMC with 2  $\mu$ M of pD1, pD2 and pD34 then stained with pD2 and pD3/4 tetramers on either the 14th (a) or 20th (b, c) day after stimulation. The highly cross-reactive populations against pD2 and pD3/4 apparent in the acute phase with high frequency (left) but in the convalescent sample with very low frequency or not detectable when more serotype specific populations have appeared (right).

Interestingly, in patient MD 893, after pD2 stimulation, there was a single population of cross-reactive cells acutely but in convalescence there were two population of cells: one had pD3/4 serotype specificity (i.e. strong binding of pD3/4 and weak binding of pD2 tetramers) and the other had pD2 serotype specificity. (Figure 5.1-c). These data suggest there are differences between acute and convalescent samples in terms of the repertoire of dengue NS3<sub>133-142</sub> specific T cells that proliferate in response to pD2 stimulation in vitro.

In addition, the double tetramer staining is a competition assay and can be considered as a measure of relative avidity between the two competing tetramer peptides than of absolute serotype specificity. Therefore, we went on to generate T cells clones to further examine the fine phenotypic differences between dengue NS3<sub>133-142</sub> specific T cells in acute and convalescent samples.

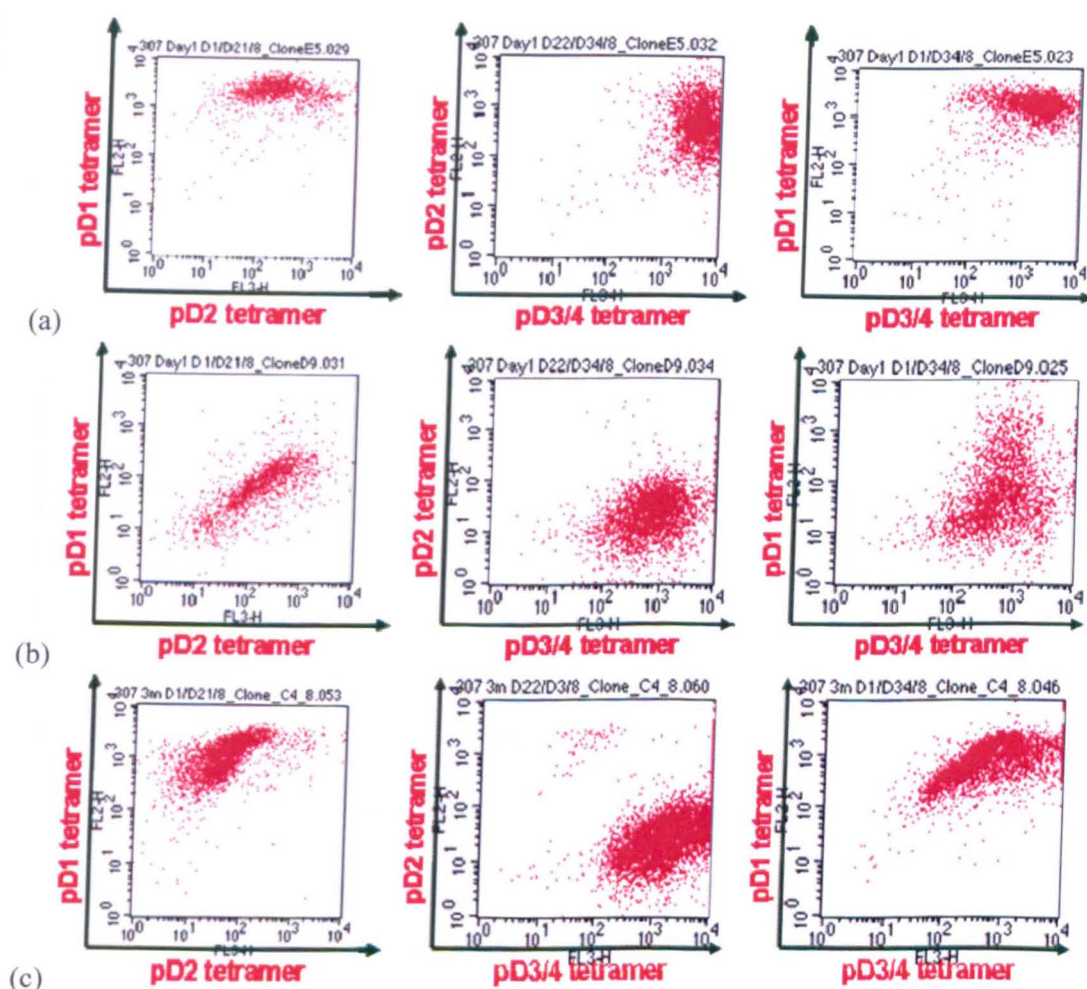
### **5.2.2 Dengue specific cross-reactive CTL clones.**

Dengue specific Cytotoxic T Lymphocyte clones were generated by the limiting dilution of T cell lines generated as described in chapter 2. Briefly, CTL lines from three above donors (stimulated with each of the 3 dengue NS3<sub>133-142</sub> peptide variants – acute and convalescent) were plated out in flat – bottom 96-well microtitre plate at a concentration of 1 cell/well in cloning mixture containing irradiated mixed allogeneic PBMCs, irradiated autologous peptide –pulsed BCLs, phytohaemagglutinin and 10% pooled human AB serum in RPMI. On day 4, 50 U of recombinant interleukin-2 per ml of 10% pooled human AB serum in RPMI was added. Wells demonstrating sufficient growth and cell density (very obvious



on microscopy of flat-bottomed plate) at each 7-day period were screened for their specificity using dengue NS3<sub>133-142</sub> tetramer staining and selected clones were expanded into 48-well (then 24-well) plates tissue culture plates with autologous, irradiated, peptide-pulsed EBV-transformed B-lymphoblastoid cells (B-LCL). An example of dual tetramer staining for a diverse selection of the clones was shown in Figure 5.2.

Figure 5.2: An example of dual tetramer staining for clone selection.



Candidates of dengue specific CTL clones from the limiting dilution of T cell lines were co-stained with pD1, pD2 and pD3/4 tetramers and CD8 antibody. The plots are gated on CD8 positive cells. (a) clone E5: cross-reactivity to pD1 – pD2 and pD3/4, (b) clone D9: pD3/5 serotype specific, (c) clone C4-8: partially cross-reactive to pD1 and pD3/4.

The expanded clones were cultured for 14 days and were screened for their specificity using pD2 and pD3/4 tetramer staining.

Specific clones were maintained by weekly restimulation with autologous, irradiated, peptide-pulsed EBV-transformed B-lymphoblastoid cells (B-LCL) and also could be kept frozen and stored in liquid nitrogen. Specific clones were studied for their lytic activity in a CTL assay and their cytokine expression profile measured by cytokine beads assays and intra-cellular Cytokine Staining assays (ICS).

Although there were populations of CTL lines that showed tetramer binding positive on either 14<sup>th</sup> or 20<sup>th</sup> day after stimulation, except BC307, specific cytotoxic T Lymphocyte clones could not be generated from MD 856 or MD 893.

Table 5.2: Dengue HLA-A11 restricted NS3<sub>133-142</sub> specific CD8+ T cell clones.

<i>CD8+ T cells clones from:</i>	<i>Acute Sample</i>	<i>Convalescent Sample</i>
pD1 stimulated	1	4
pD2 stimulated	3	0
pD3+4 stimulated	1	7
<b>Total</b>	<b>5</b>	<b>11</b>

### 5.2.3 Specificity of CTL clones using CTL lysis assays.

To determine the specificity of the 16 clones, we tested their capacity to recognize peptides corresponding to the three variant NS3<sub>133-142</sub> epitopes by using CTL lysis assays. CTL lysis assays were performed using standard <sup>51</sup>Chromium release assays. Briefly, HLA-A11 matched B-cell lines (BCL) were labeled with <sup>51</sup>Chromium for 1 hour, and then washed three times: target cells were then



divided and pulsed with each of a panel of three variants of NS3<sub>133-142</sub> peptide at different concentrations (0.1, 1 and 10  $\mu$ M). After another hour of incubation at 37°C, the peptide solution was then washed off and cells were counted and co-cultured with CTL clones at appropriate effector to target (E: T) ratios (10:1) in 96 well plates. The plates were incubated at 37°C for 4 hours, supernatants were harvested and radioactivity measured using a Beta-plate counter.

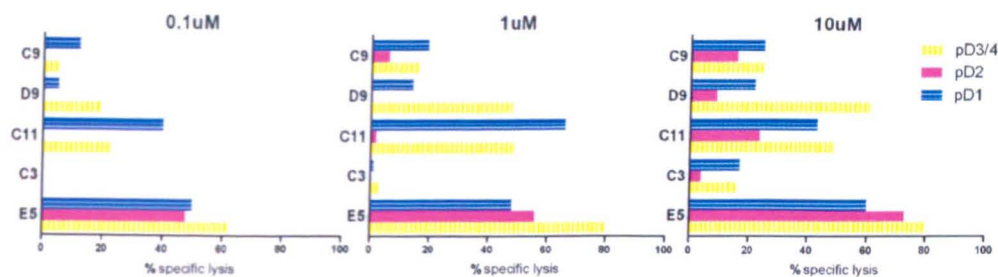
CTL clones generated by limiting dilution of short-term T cell lines derived from both acute and convalescent PBMC stimulated with either PD1, pD2 or pD3/4 had a heterogenous range of cytolytic activity. The percentage of lysis of these clones to target cells pulsed with either of three variants of NS3<sub>133-142</sub> peptide at 0.1  $\mu$ M, 1  $\mu$ M and 10  $\mu$ M are shown in Figure 5.3.

#### **Interpretation of T cell clone reactivity**

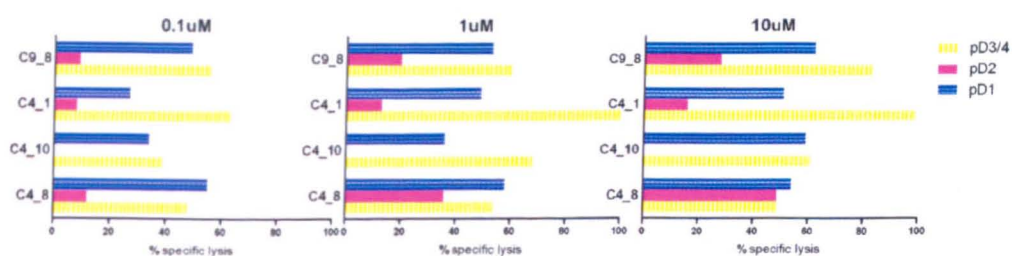
The spectrum of cross-reactivity displayed by different CD8<sup>+</sup> T cell clones is summarized in Table 5.3. Overall, most CD8<sup>+</sup> clones recognized at least 2 peptide variants of the NS3<sub>133-142</sub> epitope. In particular, a large proportion of clones recognized the highly sequence similar NS3<sub>133-142</sub> epitopes pD1 and pD3. This was independent of the type of peptide used for stimulation- for example several T cells clones elicited by in vitro stimulation with pD2 nonetheless recognized both pD1 and pD3 in the chromium-release assay. With the exception of one clone (C3), all clones recognized the peptide used for stimulation of the PBMC.

Figure 5.3: Clones differ in specificity and cytolytic efficacy in chromium-release assays.

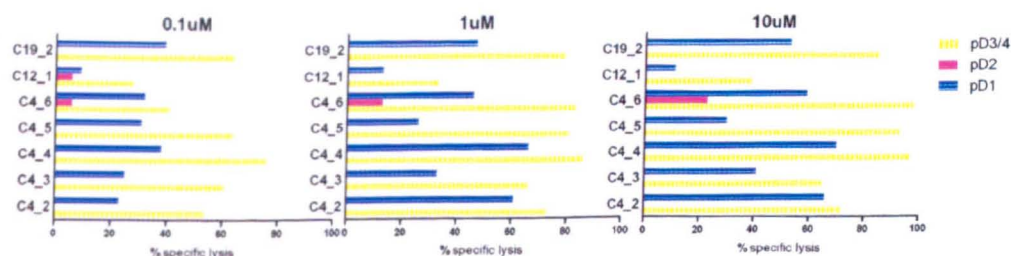
(a)



(b)



(c)



Target cells were pulsed with either pD1 (blue – horizontal lines), pD2 (pink-noline) or pD3 (yellow – vertical lines) at different concentration: 0.1uM, 1uM and 10uM. The E:T ratio was 10:1 for all clones. (a) Clones (C9, E5, C3, C11, D9) derived from acute PBMC by stimulating with pD1, pD2 and pD3/4, respectively. (b) Clones (C9\_8, C4\_1, C4\_10, C4\_8) derived from convalescent PBMC by stimulating with pD1. (c) Clones (C19\_2, C12\_1, C4\_6, C4\_5, C4\_4, C4\_3, C4\_2) derived from convalescent PBMC by stimulating with pD3/4.

Thus, pD2 stimulation elicited cross-reactive T cell clones that recognized all sequence variants of the NS3<sub>133-142</sub> epitope. It was not possible to unequivocally determine whether the T cell clones derived from this patient (BC307) represented T cells elicited de novo by the current DEN infection, or were memory cross-

reactive T cells originally elicited by a previous DEN infection and re-stimulated by the current infection.

Table 5.3: Recognition of Dengue specific Clones generated from PBMC of acute dengue infected patient by stimulating with different variants of NS3<sub>133-142</sub> epitope.<sup>(+)</sup>

Serotype Stimulation	Phase of illness	Clone name	Recognition		
			D1	D2	D3/4
<i>pD1</i>	<i>Acute</i>	C9	++	+	+
	<i>Convalescent</i>	C4_1	+++	+	+++
		C4_8	+++	++	+++
		C4_10	++	-	+++
		C9_8	+++	+	+++
<i>pD2</i>	<i>Acute</i>	E5	+++	+++	+++
		C3	-	-	+
		C11	+++	+	++
	<i>Convalescent</i>				
<i>pD3/4</i>	<i>Acute</i>	D9	+	-	+++
	<i>Convalescent</i>	C4_2	+++	-	+++
		C4_3	++	-	+++
		C4_4	+++	-	+++
		C4_5	++	-	+++
		C4_6	++	+	+++
		C12_1	+	-	++
		C19_2	++	-	+++

<sup>(+)</sup> Peptide recognition is classified by the percentage of specific lysis of B cells loaded with 1  $\mu$ M of peptide in a standard chromium release assay. +++ 50% or greater lysis, ++ between 20 and 50% lysis, + less than 20% lysis, - less than 5% or no lysis

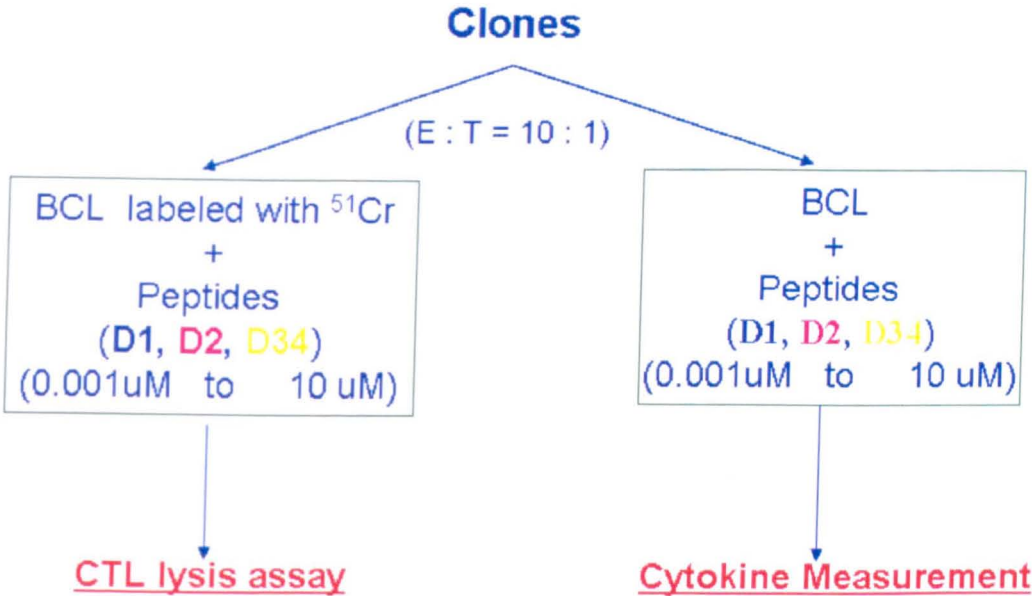
#### 5.2.4 Further functional characterization of NS3<sub>133-142</sub> specific CD8<sup>+</sup> T cell clones.

Subsets of clones were selected for further study in the basis of their peptide recognition patterns in chromium-release assay. Thus, clones E5 and C4.8 were

selected because they recognized all three of the common NS3<sub>133-142</sub> variant epitopes, with C4.8 being slightly less cross-reactive for pD2. Clones C11 and C4.6 were selected because although reactive with all NS3<sub>133-142</sub> variant epitopes at high peptide concentrations, they failed to recognize pD2 at low concentrations. Finally, clone D9 was selected because of its relatively specific recognition of pD3/4. This subset of clones were tested for their CTL activity against peptide variants across a range of peptide concentrations, including low concentrations as might be found *in vivo*. In the same experiments, further functional analyses of the clones were performed by measuring cytokines in supernatants from co-cultures of clones and target cells. A schematic summarizing the methodology used to characterize these clones is shown in Figure 5.4.

In parallel with the CTL lysis assays, we performed a duplicate set of experiments using the scheme described above but without <sup>51</sup>Cr labeling of the BCL. Supernatant from the CTL and target cell co-culture was harvested after overnight incubation, and cytokines were measured by standard Cytokine Beads Array analysis. By using this model, we are able to compare between clones in term of cytolytic activity and cytokine production activity.

Figure 5.4: Experiment to correlate and compare Cytolytic and Cytokine production activities of Clones



**Cross-reactive clone E5**

Highly cross-reactive clone E5 maintained high levels of cytolytic activity against B-cells pulsed with all three peptide variants at concentrations as low as 0.1μM (Figure 5.5).

**Partially cross-reactive clones C11, C4.6 and C4.8**

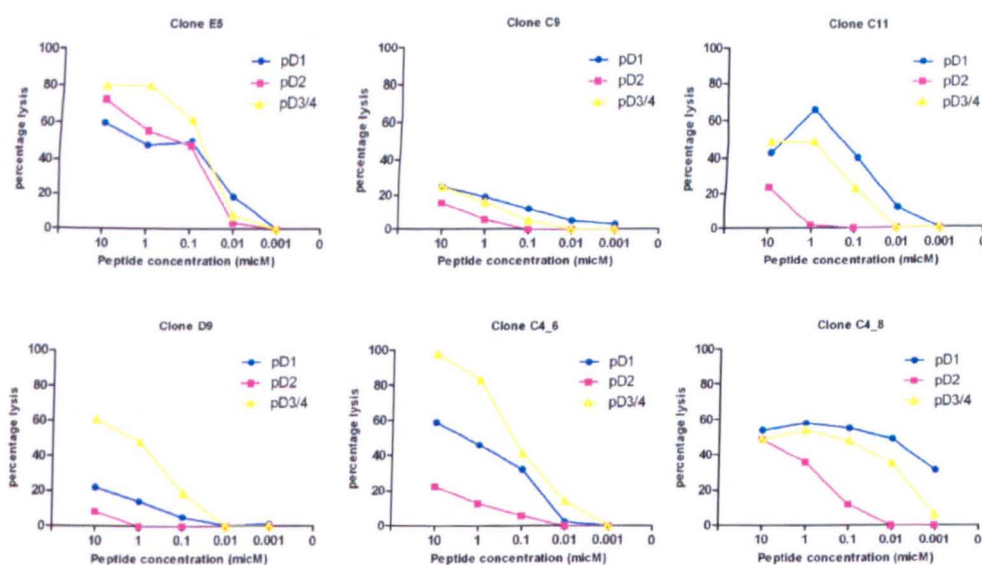
C11 (derived from acute PBMC with pD2 stimulation) showed robust killing activity against pD1 and pD3/4, but no measurable activity against pD2 at concentrations below 10 μM concentration. Clone C4.6 (derived from convalescent PBMC with pD3 stimulation) was lytic for cells pulsed with pD3/4 and to a lesser extent, pD1, but had minimal activity against pD2 (Figure 5.5). Partially cross-reactive clone C4.8 (derived from convalescent PBMC with pD1

stimulation) showed good lytic activity against cells pulsed with pD3/4 and pD1 but activity against pD2 decreased in a peptide concentration dependent manner.

### Relatively serotype specific clones

Serotype specific clone D9 was lytic for target cells pulsed with its cognate peptide (pD3/4) but had minimal or absent activity against pD1 and pD2 respectively at peptide concentrations less than 10  $\mu$ M (Figure 5.5).

Figure 5.5: Cytolytic activity of Highly Cross reactive Clone (E5), Serotype Specific Clone (D9) and Partially Cross Reactive Clones.



*Target cells were pulsed with either pD1 (blue – round lines), pD2 (square line) or pD3 (yellow – triangle lines) at different concentration. The E:T ratio was 10:1 for all clones*

These data on lytic activity amongst T cell clones suggested *heterogeneity* in the way these T cells responded to NS3<sub>133-142</sub> peptide variants. To further explore functional differences between the clones, we analyzed cytokine responses by clones in response to specific antigenic stimulation, especially focus on IFN $\gamma$  and

TNF $\alpha$  as they have been implicated in the pathogenesis of the capillary leak syndrome in DHF.

**Patterns of cytokine release from CTL clones correlate with cytolytic activity.**

For many clones, IFN $\gamma$  and TNF $\alpha$  production by the clones correlated with their cytolytic activity. The higher cytolytic activity does, the higher of cytokine release is.

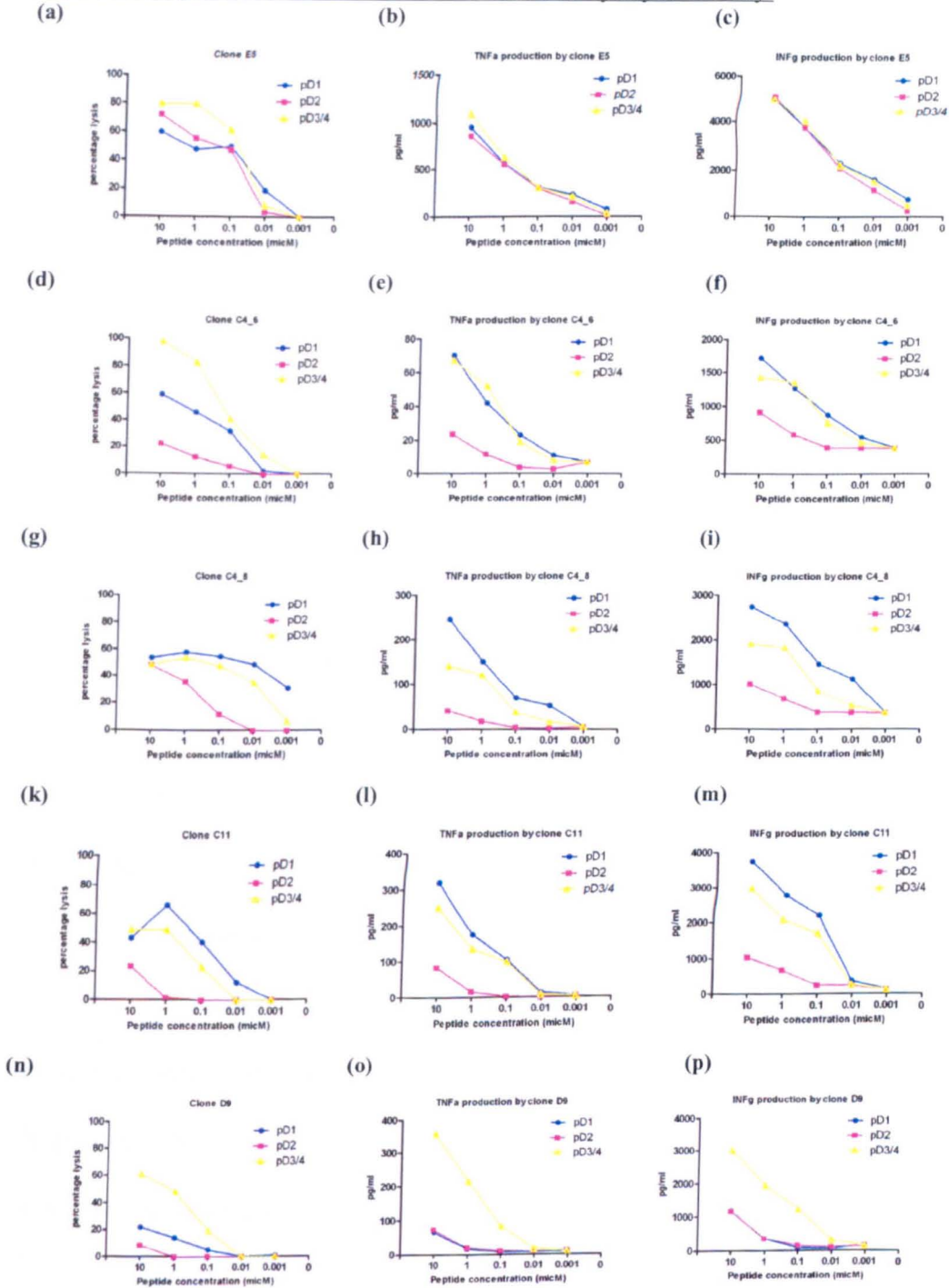
For example, clone C4.6 had highest lytic activity against pD3/4 (Figure 5.6 d-f) and also produced higher levels of TNF $\alpha$  and IFN $\gamma$  after stimulation with pD3/4 peptide.

Cytokine secretion profiles are consistent with CTL phenotype for partially cross-reactive T cell clones 4.6, 4.8 and C11. Shown are concentrations of TNF $\alpha$  and IFN $\gamma$  in supernatants of co-cultures containing peptide-pulsed target cells and CD8 T cell clones 4.6 (Figure 5.6 d-f), 4.8 (Figure 5.6 g-j) and C11 (Figure 5.6 k-m). In these assays, TNF $\alpha$  and IFN $\gamma$  were good correlates of the T cell clones lytic activity.

Serotype specific clone D9 showed intermediate activity and produced TNF $\alpha$  and IFN $\gamma$  against its cognate peptide (pD3/4) and failed to recognize target cells pulsed with other peptide variants and they not produced cytokines. (Figure 5.6 n-p).



Figure 5.6: Cytokine release from CTL clones and Cytolytic activity.



Target cells were pulsed with either pD1 (blue – round lines), pD2 (square line) or pD3 (yellow – triangle lines) at different concentration. The E:T ratio was 10:1 for all clones. Cytolytic activity per peptide concentration of target cells were shown on left panel, cytokine release activity per peptide concentration of target cells were shown on middle (TNF $\alpha$ ) and right( IFN $\gamma$ ) panel. (a-c) Highly cross-reactive clone E5 showed good lytic activity against cells pulsed with all three peptide variants (0.01  $\mu$ M). (d-m) Partially cross – reactive clones. (n-p) Serotype specific clone.

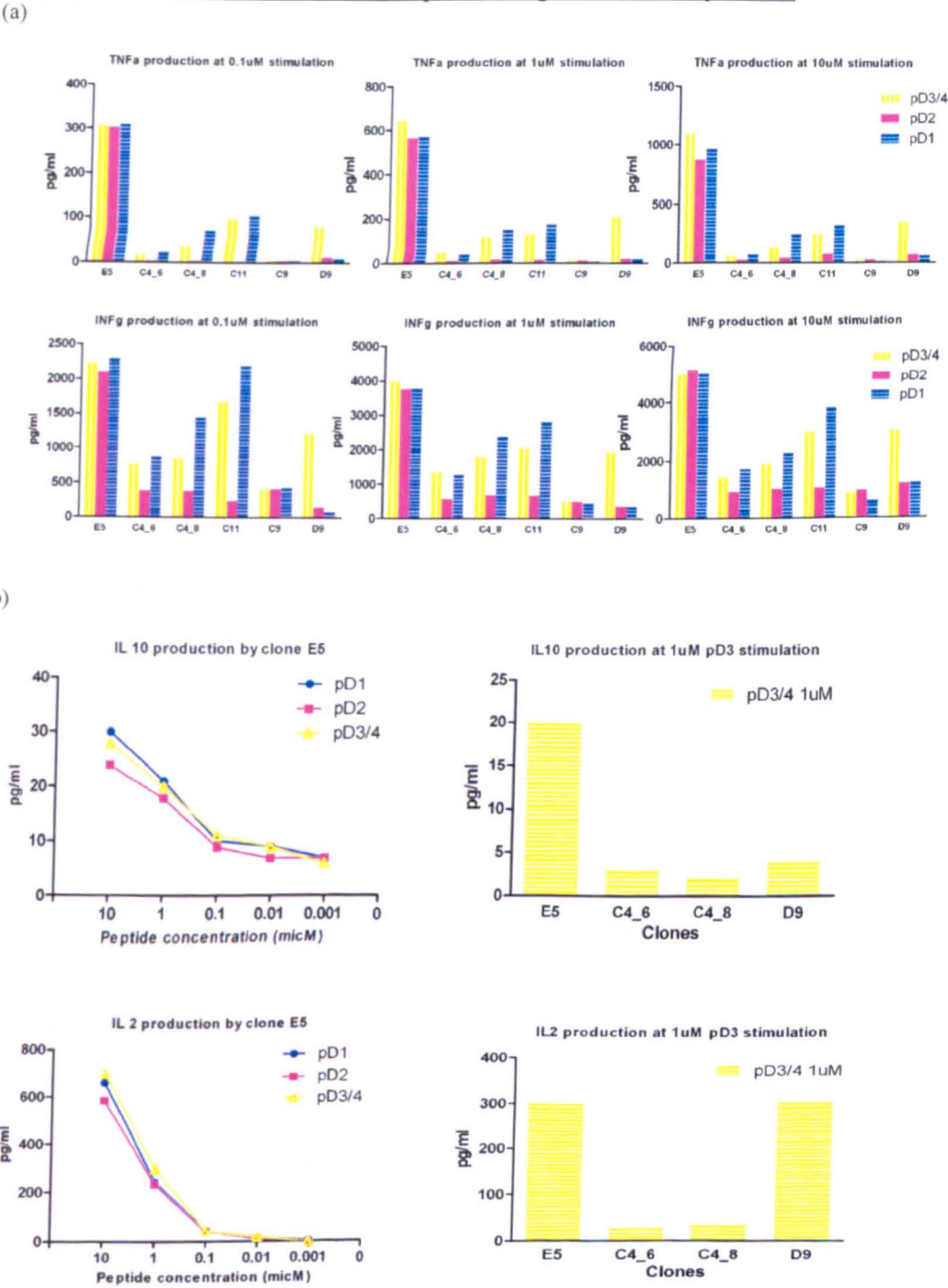


**Cross-reactive CTL clones produce higher levels and different patterns of cytokine release than serotype-specific CTL**

A feature of the highly cross-reactive clone E5 was that it consistently produced higher levels of TNF $\alpha$  and IFN $\gamma$  than the other clones when stimulated with different peptide variants across a range of peptide concentrations (Figure 5.7.a). Clones E5 and D9 produced similar quantities of IFN- $\gamma$  when stimulated by cognate peptide at high concentrations. At lower concentrations cross-reactive clone E5 produces up to twice as much IFN- $\gamma$  than serotype specific D9 or partially cross-reactive clones. Clone E5 also produces up to 4 times more TNF $\alpha$  than serotype specific D9 or partially cross-reactive clones (Figure 5.7.a). These data suggest that clone E5 is broadly able to recognize NS3<sub>133-142</sub> variant peptides and in response to cognate antigen stimulation secretes relatively high levels of pro-inflammatory cytokines.

To explore whether other patterns of cytokine secretion were associated with these T cell clones, concentrations of IL-4, IL-6, IL-2 and IL-10 were measured in culture supernatants. Along with TNF $\alpha$  and IFN $\gamma$ , clone E5 produced relatively high levels of IL-10 and IL-2 whereas the other clones secreted either very low levels or not all (Figure 5.7.b and c).

Figure 5.7: Cross-reactive CTL clones produce higher levels of Cytokines



(a)  $TNF\alpha$  and  $IFN\gamma$  production of clones at different concentration (0.1 to 10  $\mu M$ ) of different peptide variants (pD1 – blue horizontal lines; pD2 – pink no line; pD3/4 – yellow vertical lines). (b) IL 10 production by highly cross – reactive clone E5 when incubated with different peptide variant - pulsed target cells at different concentration (0.1 to 10  $\mu M$ ) of peptide (left). IL-10 was produced in high levels by clone E5. Other clones produced IL-10 in only very low levels if at all (right). (c) IL2 production by these clones.

**Intracellular cytokine secretion in antigen-stimulated clones**

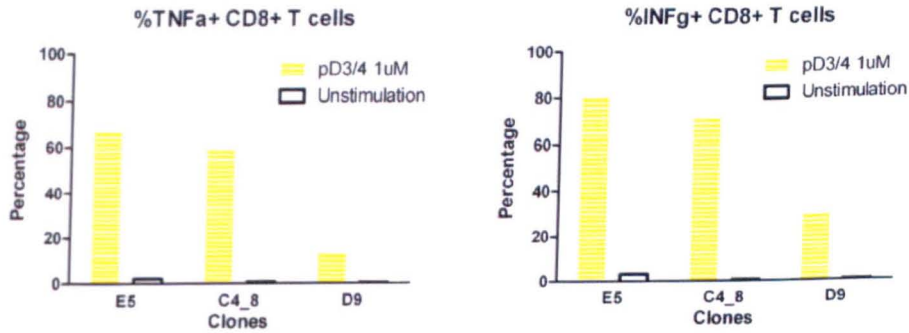
We had thus far shown that T cell clones from a patient with secondary dengue were heterogenous with respect to their lytic activity for target cells and secretion of cytokines previously associated with dengue pathogenesis. To better understand whether the increased cytokine secretion by the cross-reactive clone E5 was a result of more cells secreting cytokine, or perhaps more cytokine secreted per cell, we performed intracellular cytokine staining for TNF $\alpha$  and IFN $\gamma$  on antigen stimulated clones. High cross-reactive clone (E5), partially cross-reactive clone (C4\_8) and serotype specific clone (D9) were stimulated with 1 $\mu$ M of different peptide variant pulsed HLA-matched BCL and incubated for 6 hours in the presence of brefeldin A. Cells were washed with FACS buffer and were fixed and permeabilized with FACSTM permeabilizing solution (BD). This was followed by a wash and intracellular staining with antibodies for TNF $\alpha$ \_FITC, IFN $\gamma$ \_PE, CD8+APC and pD3/4QR then analyzed by FACS machine (chapter 2).

The frequency of TNF $\alpha$ + CD8+ T cells and IFN $\gamma$ +CD8+T cells following stimulation with 1 $\mu$ M pD3/4 to high cross-reactive clone (E5) was higher than those of partially cross-reactive clone (C4\_8) and serotype specific clones (D9). (Figure 5.8, Table 5.4 and Figure 5.9).

Table 5.4: Double intracellular staining for  $\text{TNF}\alpha$ ,  $\text{IFN}\gamma$  and  $\text{CD8}^+$ . Cytokine expression following stimulation with  $1\mu\text{M}$  of different peptide variants to high cross-reactive (E5), partially cross-reactive (C4\_8) and serotype specific clones (D9).

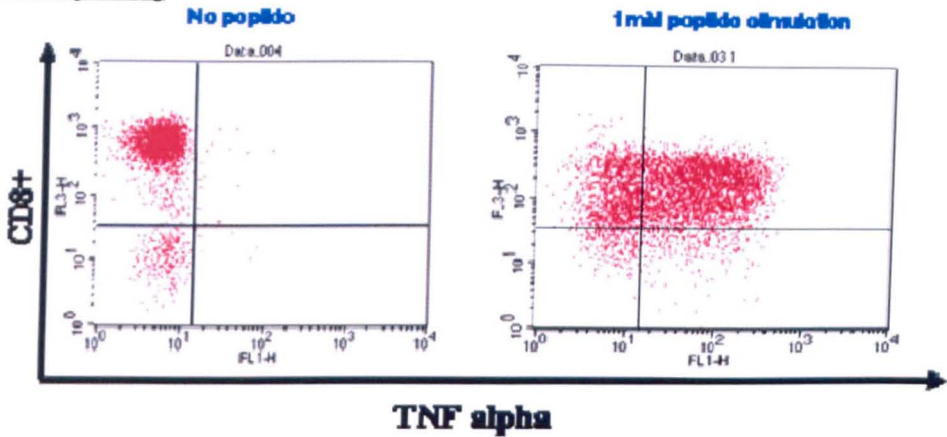
Clones		% positive $\text{CD8}^+$ CTL	
		$\text{TNF}\alpha$	$\text{IFN}\gamma$
C4_8	Unstimulation	0.56	0.48
	pD1	61	72
	pD2	13	29
	pD34	59	71
D9	Unstimulation	0.25	0.22
	pD1	3	10
	pD2	5	11
	pD34	13	29
E5	Unstimulation	2	3
	pD1	52	72
	pD2	37	51
	pD3/4	67	80

Figure 5.8: Intracellular cytokine secretion in antigen-stimulated clones



Percentage of  $\text{TNF}\alpha^+$   $\text{CD8}^+$  T cells (left) and  $\text{IFN}\gamma^+$   $\text{CD8}^+$  T cells (right) following stimulation with with  $1\mu\text{M}$  pD3/4 to high cross-reactive (E5), partially cross-reactive (C4\_8) and serotype specific clones (D9).

Figure 5.9: An example of Intracellular cytokine secretion in antigen-stimulated clone by FACS ICS plotting



*TNF $\alpha$ + CD8+ T cells following stimulation with with 1  $\mu$ M pD3/4 to high cross-reactive clone E5 (Right) and no stimulation (Left).*

### 5.3 Discussion

In this chapter, we have performed ex vivo characterization of NS3<sub>133-142</sub> specific T cells by stimulation of PBMCs then identified and characterized a panel of NS3<sub>133-142</sub> specific CD8+ T cells clones. A feature of the T cell clones was their heterogeneity in lytic activity and cytokine secretion when stimulated with NS3<sub>133-142</sub> variant epitopes.

As we observed that short-term stimulation of PBMCs with epitope peptides produced large expansions in the tetramer positive populations, in order to assess the fine specificities of dengue-specific CTL with limited cell numbers, we grew short-term CTL lines by stimulating PBMCs with each of the A11 NS3<sub>133-142</sub> epitope variants.

We showed that the highly cross-reactive populations against pD2 and pD3/4 apparent in the acute phase with high frequency but in the convalescent sample

with very low frequency or not detectable when more serotype specific populations have appeared.

We considered such CTL – those recognizing both pD2 and pD3/4 – to be highly cross-reactive given their equal recognition of both tetramers despite significant sequence variation. CTL recognizing pD1 usually recognized pD3/4 – it might be explained as a reflection of their greater homology. Cells showing high cross-reactivity between pD2 and pD3/4 always cross-reacted with pD1. Interestingly, in convalescent samples, these highly cross-reactive CTL were at low frequency or undetectable.

The previous chapter showed that *ex vivo* staining of PBMC from patient BC307 demonstrated equally high levels of CD38+ staining among all tetramer positive cells in the acute phase. CD38 expression was greater on those highly cross-reactive T cells than partially or non cross-reactive T cells. This suggested that highly cross-reactive T cell populations are more activated than cells exhibiting low levels of cross-reactivity. Therefore, we went on further assessment the cross-reactivity of dengue-specific CTL by exploring the cytotoxic characteristics of CTL clones.

Five CTL clones were isolated from PBMCs in acute sample from donor BC307 by stimulation with each of three variants of NS3<sub>133-142</sub> peptide. In convalescent cells from the same donor only pD1 and pD3&4 stimulation generated NS3<sub>133-142</sub> specific clones (11 clones).

CTL clones stimulated with each variants of peptide showed were either serotype specific (i.e. only strong recognition for a single variant peptide tetramer), partial

cross-reactivity (both showing strongest recognition for pD3/4 and differing recognition of pD1 or vice versa) or highly cross-reactivity between pD2 and pD3/4 (pD1) tetramers. Highly cross-reactive clone showed high levels of cytolytic activity at low peptide concentrations with all three peptide variants in a standard chromium release assay. Partially cross-reactive clones varied in the extent to which they recognized the epitope variants on pulsed B-cells in CTL lysis assays. They were grown from lines derived from acute and convalescent samples. Partially cross-reactive clones derived by either pD1 or pD3/4 stimulation showed strongest lytic activity against cells pulsed with corresponding sequence of NS3<sub>133-142</sub> peptide that stimulated the memory cells in PBMC (cognate stimulator), respectively but varied lower activity against other reciprocal peptide. All cross-reactive or partially cross-reactive clones maintained lytic activity against B-cells pulsed with peptide concentrations as low as 0.01  $\mu$ M. In this study, there was only one serotype specific clone (E5) that showed intermediate activity against its cognate (stimulation) peptides but failed to recognize target cells at these low levels.

Our cloning phenotypes are similar to our previous finding that large expansions of highly cross-reactive CTL (binding to both pD2 and pD3/4 tetramers equally well) were seen in pD2 stimulated cultures of acute but not convalescent PBMC samples from the same patient. They are also comparable with results of *ex vivo* staining of PBMC from patient BC307 that could showed pD1-pD3/4 cross-reactive cells at 3 weeks after illness but not pD2-pD3/4 cross-reactive cells.

In this study, we could isolate only one good D3/4 serotype specific clone (D9) from acute PBMC with NS3<sub>133-142</sub> peptides. Clone C3 was another D3/4 serotype specific but with low lysis activity (5%) at concentration of 1  $\mu$ M of peptide in a standard chromium release assay; at higher concentration of stimulation, it showed approximately 20% cross-reactivity lysis activity against both pD3/4 and pD1, 5% lysis activity against pD2. It would emphasize that this C3 clone was generated by challenge with pD2. Challenge with pD2 could expand three clones from acute PBMCs but not convalescent; among those, one was highly cross-reactive against all four serotypes (E5), one was partial cross-reactivity (C11) and one was serotype specific with low lysis activity (C3). Whereas, almost the rest of clones that stimulated by either pD1 or pD3/4 were partial cross-reactivity against only those two peptides with relatively equal magnitudes. Regarding our experiment as an alternate way of T cell clone expansion *ex-vivo* in sequential challenge from acute secondary dengue infected PBMCs, “original antigenic sin” phenomenon have been seen in pD2 stimulation. These patterns of dengue specific responses also suggested that this patient might be acutely infected by DEN-3 or Den-4 serotype. In addition, original antigenic sin could explain why highly cross-reactivity and serotype specific clones were not likely generated from challenging convalescent PBMCs. It is possible that serotype specific precursor memory CTLs are in very low frequency in convalescent samples in comparison with the partially cross-reactive memory CTLs.

Recently, my colleague Dr Tao Dong established a clone from an acute dengue patient as part of our collaborative research project, also found a similar



phenomenon in three highly cross-reactive clones of one 10-year-old dengue 2 infected child (MD1413). These clones were grown from short term lines derived from acute samples but not convalescent samples, and produced high levels of both type 1 and type 2 cytokines than serotype specific clones (Dong, Moran et al. 2007). Therefore, we have demonstrated that in hospitalized Vietnamese patients with acute dengue virus infection, a population of high cross-reactivity CTLs between all four dengue serotypes and a significant degree of activation could be detected in peripheral PBMCs. High cross-reactivity CTL clones also could be expanded by ex-vivo challenging with HLA-A11\_NS3<sub>133-142</sub> pD2, which is possibly not the current serotype infection (suggested DEN-3/4 serotype as discussion above).

In this study, we reported that the highly cross-reactive clone grown from acute samples may secrete IL-10 and produce more high levels of TNF $\alpha$  even at low peptide concentrations (0.01  $\mu$ M), whereas serotype specific clones do not secrete any IL-10 and produce much less TNF $\alpha$ , IFN $\gamma$ .

As discussed in Chapter 3, Type 1 cytokines (IFN- $\gamma$ , TNF- $\alpha$ ) exhibit vasoactive properties and are likely to participate in the pathogenesis of vascular leak. In addition TNF- $\alpha$  can mediate activation-induced cell death in some T cells (Zheng, Fisher et al. 1995) and has been implicated in peripheral T-cell deletion (Speiser, Sebzda et al. 1996; Sytwu, Liblau et al. 1996). Type 2 cytokines such as IL-10 also have been implicated in the pathogenesis of severe dengue (Mustafa, Elbishbishi et al. 2001; Perez, Garcia et al. 2004). IL-10 can be produced by distinct CD4 $^{+}$  and CD8 $^{+}$  T cell populations and has the ability to suppress T cell function (Tanchot,

Guillaume et al. 1998; Pestka, Krause et al. 2004). The net effect of interactions and balance between proinflammatory and anti-inflammatory molecules over time determines the nature of the immune response in individual patients. It could be postulated that certain type 2 cytokines such as IL-10 produced in acute disease by a subset of highly cross-reactive CTL might exert an inhibitory effect on dengue specific effector T cells.

These findings could be interpreted as implicating highly cross-reactive CTLs in the pathogenesis of severe dengue disease through differential release of vasoactive and pro-inflammatory factors. We propose that during the acute illness of secondary infection with a heterologous strain of dengue virus, high cross-reactive CTLs may be generated for some epitopes that produce high levels of TNF $\alpha$  and IL-10 and may contribute to plasma leakage and cell death.

Our study had several limitations. Although there were populations of CTL lines that showed positive tetramer binding on either the 14th or 20th day after stimulation, with the exception of BC307, dengue specific Cytotoxic T Lymphocyte clones could not be generated from patients MD856 and MD893. In our experiment, T cell clones were generated from the portion of PBMCs (approximately 10<sup>7</sup> cells for each cloning process) that were taken from peripheral blood samples at a certain time point during the acute phase and during the follow-up phase of one severe patient, and therefore might not be representative of other time points in the illness process. More generally, the patients who were admitted into our study are not representative of the whole population of individuals with dengue in the community. The cloning progress was also likely influenced by

varied factors that might impact on the generation of the clones, e.g. cell concentration, culture conditions, timing of stimulations.

To further understand the role of highly cross-reactive CD8 T cell that produce high levels of cytokines in the pathogenesis of dengue, further work is needed. Such studies should include larger numbers of patients across a range of disease severities that are followed early in their illness with specific tetramer reagents until convalescence.

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# **CHAPTER 6**

## **DISCUSSION**

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Transient increase in vascular permeability is the cardinal pathological feature in DHF that results in the leakage of plasma to the interstitial space. Many factors including total virus burden, viral virulence, host immune response (Bethell, Flobbe et al. 1998) (Leitmeyer, Vaughn et al. 1999) (Vaughn, Green et al. 2000), and genetic predisposition (Stephens, Klaythong et al. 2002) have been implicated in the plasma leakage. The findings in my thesis strongly implicates vaso-active cytokines such as TNF $\alpha$ , soluble TNF receptors, IFN $\gamma$ , IL-2, IL-6, IL-10 and possibly, the imbalance between these proinflammatory and anti-inflammatory cytokines of immune system in the pathogenesis of dengue infection (Kurane, Innis et al. 1991) (Hober, Poli et al. 1993) (Hober, Delannoy et al. 1996) (Bethell, Flobbe et al. 1998) (Hober, Nguyen et al. 1998) (Green, Vaughn et al. 1999) (Braga, Moura et al. 2001) (Perez, Garcia et al. 2004).

For many years, the prevailing mechanism proposed to link immunological cross-reactivity with the pathogenesis of severe disease has been antibody-dependent enhancement (ADE) (Halstead 1989) (Morens 1994), in which partially cross-reactive antibodies generated against one dengue serotype fail to provide protection against other serotypes but enhance macrophage infection by heterologous virus. The mechanism of action of ADE is thought to involve formation of complexes of dengue virus and pre-existing, non-neutralizing antibody against a different dengue serotype; these complexes is then endocytosed by Fc receptor bearing cells (such as monocytes) (Littaua R 1990). This allows for the infection of greater numbers of cells, which can lead to a greater viral load,

particularly early in infection. High viremia titres during dengue infection, peaking 2 days before defervescence, have been shown to correlate with progression to DHF at defervescence (Vaughn DW 2000) (Libraty DH 2002).

Recent reports have linked cross-reactive cellular immune responses to dengue virus with pathogenesis (Zivny, DeFronzo et al. 1999) (Mongkolsapaya, Dejnirattisai et al. 2003). Using HLA class I peptide tetrameric complexes (“tetramers”) to identify HLA-A11-restricted CD8<sup>+</sup> T-cells specific for an immunodominant epitope in the dengue protein NS3<sub>133-142</sub> that varies between dengue virus subtypes, it was observed that in acute secondary dengue infection with a particular serotype, low affinity T-cells were preferentially expanded which showed greater specificity for NS3 variant epitopes from previous infecting serotypes, an immunological phenomenon termed “original antigenic sin”. It was proposed that these cross-reactive cytotoxic T-cells (CTL) which had been raised against a previous dengue strain would be less efficient in eliminating newly encountered dengue virus serotypes, and that this could lead to increased virus replication and more severe disease (Mongkolsapaya, Dejnirattisai et al. 2003)

In my thesis, I provide further evidence of the potential pathogenic role for CTL in Vietnamese patients with acute dengue virus infection, a population of high avidity CTLs that show substantial cross-reactivity between all four dengue serotypes and a significant degree of activation can be detected. However, these highly activated cross-reactive cells cannot be detected in convalescent samples. The failure to expand these cells from convalescent samples suggests they are absent or greatly

reduced in number by this time point. This may be a consequence of the large ADE-augmented antigen load combined with the high avidity of the T cells leading to over-activation, T cell exhaustion and cell death presumably as a consequence of activation-induced cell death (Green, Droin et al. 2003). Newly generated CTLs therefore dominate that show a much greater degree of serotype specificity.

When CTL clones are expanded from either acute or convalescent samples, significant differences in the cytokine profile are seen: cross-reactive clones grown from acute samples may secrete IL-10 and produce high levels of TNF $\alpha$ , whereas serotype specific clones do not secrete any IL-10 and produce much less TNF $\alpha$  and IFN $\gamma$ . These findings have significant implications for understanding the role of highly cross-reactive CTLs in the pathogenesis of severe dengue disease through differential release of vasoactive, pro-inflammatory factors and possibly anti-inflammatory factors.

Discussion surrounds the precise roles of antibody and cellular immunity in term of overall immune system in dengue infection. Antibody enhancement of secondary infection facilitates viral infection of cells leading to high viraemia, antigen loading of antigen-presenting cells and a consequently vigorous cellular immune response. My observations outlined here suggest that not only the magnitude, but the specific “style” of the immune response could be of significance. Memory CTL demonstrating both serotype-cross reactivity (a pre-requisite for expansion from the memory pool in secondary infection) and higher levels production activity of inflammatory cytokines than their serotype specific

peers. In vivo this may contribute to the level of those vasoactive cytokines shown to have an association with plasma leakage and disease severity. Moreover, as discussion about Carr's experiment (Carr, Hocking et al. 2003) in chapter 3, vasoactive cytokines released from cellular immune response possibly enhance further the permeability of activated endothelial cells that were pre-exposed by TNF-alpha released from dengue virus-infected monocytes in early phase of infection. Those former cytokines possibly act as another pulse of vaso-active factor(s) in the "later phase" of the illness-immune responses and reinforce the circulation system overdrive their threshold of compensation, thus shock syndrome is developed. This hypothesis could be applied to the manifestation of plasma leakage in dengue haemorrhagic fever clinically. All DHF patients had progressively increased endothelial permeability with variety of degree when present at the clinics and the peak of plasma leakage occurs late in the course of disease, usually after the decline in fever, at day 4 to day 5 after onset. It is possibly that plasma leakage pathogenesis is a complex, multifactorial process, in that, in early phase of dengue infection (i.e. viremia and fever), cytokine cascades released mainly by the activation of monocytes/macrophages and possibly by complement activation, the interaction of other vaso-mediators such as histamine, tissue plasminogen activator (tPA), macrophage migration inhibitory factor (MIF). In later phase, cytokine response is clearly closely linked to T-cell activation and the complex interaction of antibody - dependent enhancement – viral replication - T cell activation - cytokine release interplays of self-reinforcing pathological



events which ultimately increases further vascular permeability and leads circulatory failure.

In term of cardinal feature of plasma leakage and immunopathogenesis in dengue infection, we postulated that dengue disease exists as a continuous spectrum of clinical manifestation rather than classical distinct clinical entities such as dengue fever, dengue haemorrhagic fever and dengue shock syndrome. A new dengue classification scheme based on an understanding of the pathophysiology is therefore necessary in order to help in defining the direction of epidemiology and pathogenesis research, and to improve clinical diagnosis and management.

On limitation of my studies was the relatively small number of patients to allow firm conclusions to be drawn regarding disease pathogenesis. Nonetheless these findings have potentially significant implications for understanding the role of virus-specific CTL in immunity to dengue virus infection and in the pathogenesis of severe dengue disease. Further work is needed to extend them and relate the presence or absence of such *high-avidity cross-reactive cells* to clinical spectrum.

The significant problems of multiple serotypes of dengue virus, ADE with cross-reactive antibody, serotype cross-reactive T cells are important challenges in furthering our knowledge of dengue infection. Management of dengue infection currently relies on symptomatic treatment with fluid replacement that aims to compensate the plasma leakage and resuscitation if circulation failure occurs. Radical therapy with a specific treatment, administered in the early stages of dengue infection, which prevents viral replication and thus reduces the high viral

load associated with severe disease, represents an attractive option in the fight against dengue in future. Greater understanding of the immune mechanism underlying the disease may help us design rational immune modulatory treatment and aid in the development of vaccines.

In recent years there has been considerable progress in the production of a vaccine against dengue with advanced candidates undergoing Phase II and soon Phase III clinical trials (Bhamarapravati and Sutee 2000) (van Der Most, Murali-Krishna et al. 2000) (Kochel, Raviprakash et al. 2000) (Guirakhoo, Pugachev et al. 2002). Unfortunately, knowledge that cross-reactive immune responses between dengue serotypes may play a part in the pathogenesis of severe disease has been a substantial obstacle to dengue vaccine development. It is important to assess not only the nature of the antibody response but the fine specificity, and effector function of T-cells elicited by putative dengue vaccine candidates in order to aid the development of a vaccine that produces pan-serotype protective immunity without the risk of iatrogenically excessive vaso-active cytokine release.

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# **APPENDICES**

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**Appendix 1: List of HLA-A11 predicted peptides (nonamers & decamers) of serotype 2 dengue (strain 16681)**

ID	Start Position*	Location	Subsequence Residue Listing	Type
1	36	C	GMLQGRGPLK	10 mers
2	163	preM	ITYKCPFLK	9 mers
3	318	E	KPTLDFELIK	10 mers
4	394	E	VTCAMFTCKK	10 mers
5	399	E	FTCKKNMEGK	10 mers
6	506	E	TQGSNWIQK	9 mers
7	518	E	VTFKNPHAK	9 mers
8	518	E	VTFKNPHAKK	10 mers
9	566	E	RLRMDKLQLK	10 mers
10	576	E	GMSYSMCTGK	10 mers
11	582	E	CTGKFKVVK	9 mers
12	632	E	TTVNPIVTEK	10 mers
13	665	E	GQLKLDWFK	9 mers
14	665	E	GQLKLDWFKK	10 mers
15	666	E	QLKLDWFKK	9 mers
16	835	NS1	VTRLENLMWK	10 mers
17	860	NS1	KLTIMTGDIK	10 mers
18	861	NS1	LTIMTGDIK	9 mers
19	887	NS1	RYSWKTWGK	9 mers
20	936	NS1	GVFTTNIWLR	10 mers
21	949	NS1	KQDAFCDSK	9 mers
22	1038	NS1	QTAGPWHLGK	10 mers
23	1217	NS2	GLLLRKLTSK	10 mers
24	1335	NS2	LTTLRSRTSK	9 mers

25	1335	NS2	LTTLSRTSKK	10 mers
26	1336	NS2	TTLSRTSKK	9 mers
27	1509	NS3	SQIGAGVYK	9 mers
28	1527	NS3	VTRGAVLMHK	10 mers
29	1570	NS3	VQVLALEPGK	10 mers
30	1583	NS3	AVQTKPGLFR	10 mers
31	1608	NS3	GTSGSPIIDK	10 mers
32	1692	NS3	RTLILAPTR	9 mers
33	1832	NS3	KTVWFVPSIK	10 mers
34	1863	NS3	KTFDSEYVK	9 mers
35	1990	NS3	KVDAIDGEYR	10 mers
36	2006	NS3	KTFVDLMRR	9 mers
37	2102	NS4	RLPTFMTQK	9 mers
38	2161	NS4	FLMSGRGIGK	10 mers
39	2312	NS4	ATVLMGLGK	9 mers
40	2317	NS4	GLGKGWPLSK	10 mers
41	2398	NS4	IPYDPKFEK	9 mers
42	2472	NS4	GAGLLFSIMK	10 mers
43	2521	NS5	IQEVDRTLAK	10 mers
44	2526	NS5	RTLAKEGIK	9 mers
45	2526	NS5	RTLAKEGIKR	10 mers
46	2564	NS5	KVVDLGCGR	9 mers
47	2581	NS5	GLKNVREVK	9 mers
48	2841	NS5	RVFKEKVDTR	10 mers
49	2850	NS5	RTQEPKEGTK	10 mers
50	2880	NS5	RMCTREEFTK	10 mers
51	2882	NS5	CTREEFTKK	9 mers
52	3004	NS5	GYILREVSK	9 mers
53	3004	NS5	GYILREVSKK	10 mers
54	3041	NS5	ITNHMAGEHK	10 mers

55	3075	NS5	GTVMDIISR	9 mers
56	3109	NS5	RQMEGEGIFK	10 mers
57	3137	NS5	RVGRERLSR	9 mers
58	3166	NS5	LTALNDMGK	9 mers
59	3340	NS5	GLTSRATWAK	10 mers
60	3341	NS5	LTSRATWAK	9 mers
61	3367	NS5	YTDYMPSMK	9 mers

Note: \* position from the beginning of the viral genome.

<i>capsid protein C</i>	<i>1-114</i>
<i>Pre_M</i>	<i>115-205</i>
<i>M</i>	<i>206-280</i>
<i>E</i>	<i>281-775</i>
<i>NS1</i>	<i>776-1127</i>
<i>NS2A</i>	<i>1128-1345</i>
<i>NS2B</i>	<i>1346-1474</i>
<i>NS3</i>	<i>1475-2093</i>
<i>NS4a</i>	<i>2094-2243</i>
<i>NS4b</i>	<i>2244-2491</i>
<i>NS5</i>	<i>2492_3391</i>

**Appendix 2: List of patients in the screening for Dengue serotype 2 HLA-A 11 epitope peptides (Chapter 4)**

ID	Hosp No	Age	Gender	Adm date	Day of Illness	DHF grade	Serotype	Hct	Platelet	WBC	N%	L%
BC001	18615	28	F	02-06-02	3	2	1, 4	42	142000	6100	51.8	36.3
BC303	22611	25	M	01-07-02	6	2	unknown	41	17000	5840	58.9	16.4
BC304	23403	36	F	04-07-02	4	3	unknown	42	56000	7100	75.7	23.8
BC306	31142	17	M	27-08-02	6	2	unknown	49	22600	3810	67.4	25.4
BC307	31918	17	F	04-09-02	6	2	unknown	44	48000	7500	50.3	36.1
BC310	33951	17	F	20-09-02	3	3	unknown	38	106000	4090	70.6	12.3
BC322	45756	26	M	12-12-02	3	3	unknown	52	25000	4080	50.2	25.5
BC323	46074	31	M	16-12-02	4	3	unknown	53	75000	8540	29.5	24.0
BC328	2073	15	F	05-02-03	5	1	unknown	42	36000	8430	32.0	56.0
BC330	3320	16	M	09-02-03	6	3	4	46	46500	3080	59.8	20.7
BC332	4274	16	M	13-03-03	5	3	unknown	51	45800	4870	73.7	17.2
BC357	11690	26	M	30-06-03	5	3	2	50	40000	6120	40.0	43.0
BC363	12686	31	F	15-07-03	5	3	unknown	41	36000	1400	51.0	42.0
BC375	14349	16	M	10-08-03	4	2	unknown	38	38800	3870	60.3	16.4
BC378	14576	21	M	13-08-03	5	2	2	57	49000	3800	42.2	45.5
BC380	14710	17	M	15-08-03	5	2	1	48	144000	4300	72.1	22.9
BC395	15503	23	F	26-08-03	5	2	2	46	18000	4400	36.0	55.0
BC412	16686	23	F	11-09-03	5	3	unknown	41	19100	5900	42.0	55.0
BC418	17050	21	M	16-09-03	3	2	unknown	43	155000	5990	79.2	19.0

BC419	17116	22	M	17-09-03	5	3	4	49	20100	2870	46.1	24.0
BC420	17262	21	M	18-09-03	4	2	unknown	55	24000	3600	21.0	31.0
BC423	14252	18	F	18-09-03	5	1	unknown	41	28000	1900	61.0	23.0
BC428	17644	17	M	24-09-03	6	3	4	43	27900	5980	34.7	28.9
BC437	18155	15	M	01-10-03	6	2	3	44	96600	3220	59.4	21.7
BC439	18162	15	F	01-10-03	4	2	2	40	88500	2600	34.6	39.3
BC440	18258	16	F	02-10-03	6	3	unknown	39	26000	7600	52.3	35.6
BC441	18199	21	F	01-10-03	4	2	unknown	45	20000	5100	20.0	70.0
BC444	18490	17	M	06-10-03	5	2	unknown	50	14000	3470	38.0	50.0
BC467	20919	19	F	06-11-03	4	2	unknown	41	18500	3620	33.3	36.3
BC494	23601	25	F	07-12-03	4	2	unknown	40	31000	2670	34.0	54.0
BC703	24673	15	M	19-12-03	4	2	unknown	41	55900	5850	50.8	26.5

**Appendix 3: List of patients with acute phase PBMCs using in IFN $\gamma$  ELISPOT assays against variants of NS3<sub>133-142</sub> peptides. (Chapter 4)**

ID	Hosp No	Age	Gender	Adm date	Day of Illness	DHF grade	Serotype	Hct	Platelet	WBC	N%	L%
BC337	6089	16	M	06-04-03	4	3	1	46	17300	5090	44.1	27.5
BC354	11229	17	F	24-06-03	4	2	1	50	30000	6600	72.0	21.0
BC392	15403	20	M	25-08-03	4	0	1	46	51200	1750	53.2	32.1
BC338	6758	22	F	15-04-03	3	2	2	42	33900	2490	51.0	31.1
BC378	14576	21	M	13-08-03	5	2	2	57	49000	3800	42.2	45.5
BC393	15424	17	F	26-08-03	4	3	2	49	46200	3650	68.9	21.6
BC395	15503	23	F	27-08-03	5	2	2	46	18000	4400	36.0	55.0
BC514	24292	18	F	15-12-03	6	1	2	38	44600	5600	79.0	21.0
DF110	14909	9	M	18-08-03	5	3	2	41	49200	3100	71.8	12.0
DF129	16162	8	F	05-09-03	3	3	2	41	53400	3700	56.2	37.5
DF141	16321	7	M	07-09-03	6	3	3	41	60000	8150	52.0	7.2
BC383	14927	16	M	18-08-03	6	2	4	40	31100	6100	30.7	33.8
BC390	14932	18	M	22-08-03	2	1	4	41	90000	4200	75.2	18.6
BC428	17644	17	M	24-09-03	6	3	4	34	41400	11800	26.8	36.7
DF135	16034	12	F	03-09-03	6	3	4	40	88500	11700	82.6	4.6